PHYTOCHEMICAL SCREENING, ELEMENTAL ANALYSIS AND ANTIMICROBIAL ACTIVITY ON FLOWER PART OF THREE HIBISCUS SPECIES

Dissertation Submitted to

The Tamil Nadu Dr. M.G.R. Medical University,

Chennai – 600 032.

In partial fulfillment for the award of Degree of

MASTER OF PHARMACY

(PHARMACEUTICAL CHEMISTRY)

Submitted by

M. POORNIMA

Reg.No:26106034

Under the Guidance of

Mr. M. SUGUMARAN, M. Pharm., (Ph.D.)

Associate Professor

Department of Pharmaceutical Chemistry

ADHIPARASAKTHI COLLEGE OF PHARMACY

(Accredited by "NAAC" with a CGPA of 2.74 on a Four Point Scale at "B" Grade)

MELMARUVATHUR-603 319

MAY - 2012

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CERTIFICATE

This is to certify that the research work entitled **''PHYTOCHEMICAL SCREENING, ELEMENTAL ANALYSIS AND ANTIMICROBIAL ACTIVITY ON FLOWER PART OF THREE HIBISCUS SPECIES''** submitted to The Tamil Nadu Dr. M.G.R. Medical University in partial fulfillment for the award of the Degree of Master of Pharmacy (Pharmaceutical Chemistry) was carried out by **M. POORNIMA (Reg. No: 26106034)** in the Department of Pharmaceutical Chemistry under my direct guidance and supervision during the academic year 2011-2012.

Place: Melmaruvathur
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CERTIFICATE

This is to certify that the dissertation entitled **''PHYTOCHEMICAL SCREENING, ELEMENTAL ANALYSIS AND ANTIMICROBIAL ACTIVITY ON FLOWER PART OF THREE HIBISCUS SPECIES''** is the bonafide research work carried out by **M. POORNIMA (Reg. No: 26106034)** in the Department of Pharmaceutical Chemistry, Adhiparasakthi College of Pharmacy, Melmaruvathur which is affiliated to The Tamil Nadu Dr. M.G.R Medical University under the guidance of **Mr. M. SUGUMARAN, M. Pharm., (Ph.D.),** Associate Professor, Department of Pharmaceutical Chemistry, Adhiparasakthi College of Pharmacy, during the academic year 2011-2012.

Place: Melmaruvathur
Date:

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M.POORNIMA

Dedicated To

My Beloved Parents

&

All My Friends... 

CONTENTS

LIST OF ABBREVIATIONS

^0C	:	Degree Centigrade
µg	:	Microgram
µl	:	Microliter
µm	:	Micrometer
%	:	Percentage
g	:	Gram
mg	:	Milligram
ml	:	Milliliter
cm	:	Centimeter
h	:	Hours
min	:	Minutes
pH	:	Hydrogen ion concentration
conc.	:	Concentrated
dil.	:	Diluted
rpm	:	Revolution per minute
nm	:	Nanometer
std	:	Standard
Sec	:	Seconds
v/v	:	volume/volume
R_f	:	Retention factor
KBr	:	Potassium bromide
IR	:	Infra Red
UV	:	Ultra violet
TLC	:	Thin Layer Chromatography

HPTLC	:	High performance thin layer chromatography
GC-MS	:	Gas chromatography Mass spectroscopy
AAS	:	Atomic Absorption Spectroscopy
M	:	Molarity
N	:	Normality
CFU	:	Colony Forming Unit
TIC	:	Total ion chromatogram
RT	:	Retention time
MW	:	Molecular weight
DMSO	:	Dimethyl sulphoxide
DCM	:	Dichloro Methane
EDTA	:	Ethylenediamine tetra acetic acid
DPPH	:	Diphenyl picrylhydrazyl
GABA	:	Gamma amino butyric acid
m/z	:	Mass / charge
Gram – ve	:	Gram Negative
Gram +ve	:	Gram Positive
S.aureus	:	*Staphylococcus aureus*
E.coli	:	*Escherichia coli*
A.niger	:	*Aspergillus niger*

INTRODUCTION

1. INTRODUCTION

1.1 Potentials of medicinal herbs:

Medicinal plants are used in traditional treatments to cure variety of diseases. In the last few decades there has been an exponential growth in the field of herbal medicine (Palanisamy Hariprasad. *et al*., **2011**). Medicinal plants have been used by human being since ages in traditional medicine due to their therapeutic potential and the search on medicinal plants have led the discovery of novel drug candidates used against diverse diseases. According to the World Health Organization (WHO), more than 80% of the world's population relies on traditional medicine for their primary healthcare needs (Elhoussine Derwich. *et al*., **2010**). Natural products have been a source of drugs for centuries. Ayurveda, Siddha, Unani and Folk (tribal) medicines are the major systems of indigenous medicines. Among these systems, Ayurveda is most developed and widely practised in India. Plants, especially used in Ayurveda can provide biologically active molecules and lead structures for the development of modified derivatives with enhanced activity and /or reduced toxicity.

Despite the achievement of synthetic chemistry and the advances towards rational drug design, natural products continue to be essential in providing medicinal compounds and as starting points for the development of synthetic analogues. Phytochemistry is the study of phytochemicals, a natural bioactive compound found in plants, such as vegetables, fruits, stems, bark, root, leaves and flowers that work with nutrients and fibers to act as a defense system against disease or more accurately to protect against disease. Phytochemicals produced in plants which are divided into two groups, namely primary and secondary constituents according to their functions in plant metabolism. Primary constituents comprise common sugars, amino acids,

proteins and chlorophyll while the secondary constituents consists of alkaloids, terpenoids and phenolic compounds and many more such as flavonoids, tannins and so on. Many secondary metabolites of plant are commercially important, responsible for pharmacological activities and find use in a number of pharmaceutical compounds (Krishnaiah D. *et al.,* **2009**). The potential of the phytochemicals have large scale pharmacological and biological activities such as antioxidant constituents (hydrolysable tannins, phenolic acid and flavonoids) of the plant materials for the care of health and protection from coronary heart diseases, cancer and anti-mutagenic effects. Varieties of herbaceous vegetables are protective against various diseases, particularly cardiovascular diseases. These herbaceous plants and species are harmless sources for obtaining natural antioxidants. Antioxidant constituents can delay or inhibit the oxidation of lipids and other compounds by inhibiting the propagation of oxidation chain reaction. Primarily, antioxidant effect is due to phenolic compounds such as phenolic acid, flavonoids and phenolic diterpenes and their mode of action for antioxidant compounds is due to its redox reaction properties which can absorb and neutralize free radicals by quenching singlet and triplet oxygen. Vitamins are organic substances necessary for metabolism. Human diet does not always contain the required amount of vitamins for the normal growth and maintenance of the body function and as such cannot produce enough quantity for their body metabolism, so it can be obtained from fruits, vegetables and foods. Deficiency of vitamins can cause serious human health diseases and sometimes, very small concentrations are required for maintenance of good human health (Iqbal Hussain. *et al.*, **2011**).

The major hindrance in the amalgamation of herbal medicines into modern medical practices is the lack of scientific and clinical data for better understanding of

efficacy and safety of the herbal products. So the identification of bioactive compounds in plants, their isolation, purification and characterization of active ingredients in crude extracts can be done by various analytical methods such as,

- UV Spectrophotometry.
- IR Spectrometry.
- Thin layer chromatography (TLC)
- Gel (column) chromatography)
- High performance of liquid chromatography (HPLC)
- High performance thin layer chromatography (HPTLC)
- Gas chromatography (GC)
- Mass spectrometry (MS)
- Nuclear magnetic resonance (NMR)
- Gas chromatography Mass spectroscopy (GC-MS)

Medicinal plants have great importance as food materials and in the treatment of various ailments. They provide minerals which play a vital role as structural and functional components of metalloproteins and enzymes in the living cell (Ansari. *et al.,* **2004**). Minerals are essential not only for health and performance, but more importantly for growth and development, as they play a central role in many biochemical and physiological process. Throughout the world, there is increasing interest in the importance of dietary minerals in the prevention of several diseases. Minerals are of critical importance in the diet, even though they comprise only 4–6% of the human body. Major minerals are those required in amounts greater than 100 mg per day and they represent 1% or less of body weight. These include calcium, phosphorus, magnesium, sulphur, potassium, chloride and sodium. Trace minerals are essential in much smaller amounts, less than 100 mg per day, and make up less than

0.01% of bodyweight which includes essential trace elements like zinc, iron, silicon, manganese, copper, fluoride, iodine and chromium. Deficiencies can seriously affect health and performance. The reported amounts of minerals per day are 1000 mg Calcium, 15 mg Iron and 1000 mg of phosphorus (Imelouane B. *et al*., 2011).

Studies originally showed that optimal intakes of elements such as sodium, potassium, magnesium, calcium, manganese, copper, zinc and iodine could reduce individual risk factors, including those related to cardiovascular disease for both human beings and animals. Calcium is the main component of bones and teeth, this element functions on cell membranes and on muscles, by regulating endoexoenzymes and blood pressure. Phosphorus makes up to 17% of bone mineral content, necessary for energy metabolism and Iron is an essential element for human beings and animals act as essential component of haemoglobin, it facilitates the oxidation of carbohydrates, protein and fat to control body weight, which is very important factor in diabetes (Ahmad Ali Khan. *et al*., **2009**).

1.2 Various species of Hibiscus:

1.2.1 *Hibiscus cameronii* (Fig.1.1)

It is a soft-wooded shrub 1-2 m (3-6 ft) tall. The palmate 3-5-7 lobed leaves are often serrated. The flowers are 7.5-10 cm (3-4 in) across and usually cup-shaped. The colour varies from soft apricot pink to flushed pink with spotted rose or reddish purple at base of petals and distinct veining. There is a close relationship between *H. cameronii* and the *H. rosa-sinensis* complex of varieties; they may be intercrossed, and recently scions of *H. cameronii* were grafted onto *H. rosa-sinensis* rootstock with great success. Suitable for warmer areas only, *H. cameronii* is an attractive free flowering shrub that requires light pruning occasionally. It is grown from soft tip cuttings taken in summer or by seed sown in spring, and grafted onto hardy rootstock.

A sunny aspect and light sandy soil is best. *H. cameronii* is very susceptible to root-rot in poorly drained soils.

1.2.2 *Hibiscus coccineus* (Fig.1.2)

One of the most attractive of the herbaceous perennial species of hibiscus with brilliant red, large flowers, produced during summer and autumn on erect growth to 2 m (6 ft). Leaves are long- 3 - 5 - 7 parted or compound, sometimes deeply toothed and varying from dark green to reddish purple. The flowers are 12 - 20 cm across and are produced on long pedicels in the upper leaf axils. The petals are deep red, usually not overlapping one another, revealing a contrasting light green calyx. *H. coccineus* is not as hardy as many other herbaceous hibiscus, and in colder climates it may be carried through the winter by lifting and storing the roots in a cellar or cold frame. Like the `Southern Belle' types it should be protected from strong winds, and prefers a sheltered position with full sun and a rich, well-drained soil. *Hibiscus coccineus* may be propagated by division or by seed sown in the spring.

1.2.3 *Hibiscus glaber* (Fig.1.3)

It is a branching shrub 2 - 3 m (6 - 10 ft) tall, differing from *H. tiliaceus* by leaf and petal conformation. The heart-shaped leaves are usually light green, contrasting with the bright-yellow 6 - 8 cm (2 - 3½ in) flowers. These flowers all have a purplish red basal spot and deepen to orange as they age. There is a special variation of *H. glaber* with larger, deep purplish brown foliage which is even more attractive. The plant does well, but prefers coastal conditions where it will tolerate salt-laden winds. Suitable as a specimen plant or for hedging, *H. glaber* is also useful for street plantings. It prefers full sun and a rich, well-drained soil with plenty of summer water and fertilizer. Main flowering season is summer and autumn. Prune occasionally, only

when necessary to keep a compact shape. Propagates quite readily from firm wood cuttings taken in late spring and planted in coarse river sand, preferably under glass.

1.2.4 *Hibiscus huegelii* (Fig.1.4)

It is a soft hairy shrub 1 - 2 m (3 - 6 ft) tall. Leaves are thickish, green above and below, deeply 3 - 5 lobed and parted, sometimes broadly toothed. The 12 - 16 cm blooms are produced freely in late spring and early summer. The blooms may vary from a lilac blue to a purplish red and be with or without a deep purple-red basal spot. The filaments on the staminal column are in distinct whorls near the base. Commonly known as `Desert Rose' in some places, this species requires hot, dry conditions to perform well. In warm, humid areas it suffers badly from root rot; however it may be possible to graft it onto a compatible rootstock that is more resistant. Light, sandy, well-drained soil is best, with full sun or partial shade. Propagate from seed sown in spring or by soft tip cuttings taken in summer and put in coarse river sand under mist.

1.2.5 *Hibiscus insularis* (Fig.1.5)

It is a dense bushy shrub to 4 m (12 ft). The light green foliage is small, rounded and crenate. The 6 - 9 cm blooms are produced freely in late summer and autumn. The blooms have pale lemon petals arising from a purplish crimson centre, which turn to a light purplish rose with age, both coloured blooms persisting on the bush. The bright crimson stigma pods are very pronounced for a small flower. *H. insularis* is now well established in cultivation. The plant is ideal for growing in coastal areas as it is reasonably salt resistant. It makes a fine hedge plant and windbreak. *H. insularis* prefers full sun and seems to be happy in all but poorly drained soils. Light pruning only is required to keep the plant in a nicely rounded shape. Propagate from firm wood cuttings taken in late spring and from seed sown in spring.

1.2.6 *Hibiscus manihot* (Fig.1.6)

A tall herbaceous perennial to 2 m (6 ft), sprinkled with a few pungent, bristly hairs. The leaves are deeply pinnate, lobes 5 - 9, more or less toothed. The large flowers up to 30 cm are a brilliant lemon yellow with a deep purple eye. These are produced on long pedicels on mature wood at the apex of the plant in autumn. The seed capsules produced after the flowers are very hairy, and the stiff, bristly hairs can cause severe discomfort to the unwary or allergy prone. The large blooms are easily damaged by wind and last only a few hours. *H. manihot* prefers a sunny aspect with rich moist soil. Propagated by root division or by seed sown in spring.

1.2.7 *Hibiscus moscheutos* (Fig.1.7)

The herbaceous perennial species of hibiscus native to the United States are all handsomely flowered, but except for *H. coccineus* are rather coarse plants which often seem out of place in restricted or small perennial borders. For this reason they are not encountered as frequently as one might expect from the showiness of the flowers alone. In this case *H. coccineus* was crossed with *H. militaris*, and an individual of this cross was in turn crossed with *H. moscheutos.* Progeny from this cross had flowers varying in size from 10 to 25 cm in diameter, in colours ranging from white through pink to deep red, often with a deeper eye at the centre. A similar hybridization program involving the same three parents was commenced in the early 1950s, and the results were collectively termed 'Avalon Hybrids'. Selected forms of these hybrids were crossed with *H. moscheulos* and *H. grandiflorus* to produce the giant flowering `Southern Belle' strains with blooms up to 30 cm across. These soft wooded perennials require protection from strong winds, and staking similar to dahlias is necessary to prevent wind damage. They prefer an open sunny aspect, with

rich well drained but moist soil. Liberal water and fertilizer should be applied during the flowering season to maintain the size of the flowers and to strengthen the tuberous root system, thus ensuring healthy young growth the following season. The plants begin to lose their leaves in 'autumn, but the old stems should not be cut off until they turn brown. Wait until the green disappears completely before cutting several inches above ground level. The new shoots in spring are very soft and sappy and subject to insect damage, particularly from loppers and other caterpillars. Care should be taken at this time to prevent injury to the growing tips. *H. moscheutos* hybrids are easily grown from seed sown in spring, usually flowering the first season. Fresh seed germinates readily. Selected varieties may be propagated by root division or tip cuttings. Tolerant of frosts, these hardy plants are cultivated over a much wider range of climates than the *H. rosa-sinensis* hybrids. The `Southern Belle' types attain a height of 2 m (6 ft), and the `Dixie Belle' up to 1 m (3 ft). Both are ideal for massed bed plantings or in herbaceous borders.

1.2.8 *Hibiscus mutablis* (Fig.1.8)

Small deciduous trees 5 - 6 m (15 - 20 ft) in height, covered with down and with large, downy, hairy, five to six pointed toothed leaves. The 10 - 15 cm blooms are of two forms, single and double. The single flower is cup shaped and of nice form, whilst the double is full and fluffy. The name mutablis means changeable and refers to the bloom which is pure white upon opening in the morning but during the day changes to pink and red; often the whole bush is covered with the varying shades of each colour. In Japan this hibiscus is called Fuyo and symbolises a fascinating but fickle woman. *H. mutablis* flowers in autumn, and neither single nor double forms cross with other kinds of hibiscus. This species requires an open sunny aspect with

rich well drained soil. Severe pruning in winter is recommended (about halfway) to ensure strong healthy growth and a nice rounded shape. *H. mutablis* is very hardy and will withstand quite heavy frosts. Propagation is by hardwood cuttings, about 10 - 12 mm (½ in) thick, 12 - 15 (5 - 6 in) long, taken in winter and planted in coarse river sand.

1.2.9 *Hibiscus trionum* (Fig.1.9)

Widespread in warm regions of the old world from Southern Africa, Australasia and Asia. It is an erect or straggling annual herb to 60 - 80 cm (1½ - 2 ft). Leaves are generally three lobed or parted, the lobes pinnately incised to deeply toothed. Flowers are solitary on axillary pedicels, the corolla 5 - 8 cm across, white, cream or yellow with a purple centre. *H. trionum* is cultivated to a limited extent and may be found as a casual weed in waste places and near gardens. It is commonly known as Flower-of-an-Hour, referring to the nature of the flowers which open mid or late morning and close by early afternoon. A selected strain of *H. trionum* named `Sunny Days' has been offered by seeds men recently, and has proved very popular for massed bedding displays. This handsome annual is easily grown when provided with full sun and a rich, well-drained soil. Grown from seed sown in spring.

1.2.10 *Hibiscus sabdariffa* (Fig.1.10)

Widely distributed throughout the tropics and sub-tropics, but probably native to tropical Africa where its closest relatives occur. An erect annual to biennial to 2 m, with small leaves, usually three lobed. The stems are glabrous, reddish or reddish purple. The flowers are small, about 8 cm (3 in), petals light yellow or sometimes lightly diffused with pink, with a deep red basal spot. The calyx becomes enlarged

and fleshy in fruit to 3 cm (1 %, in) long, turning bright red with an acid flavour. Seeds, leaves and shoots, as well as the calyces, are used as food, and the juice from the calyces is sometimes expressed to make a fresh or fermented drink. Although the plant is a true biennial it is much better when treated as an annual and grown from fresh seed each season. It prefers the warmer areas only and will not tolerate frost. Should be grown in a warm sunny position with rich well drained soil.

1.2.11 *Hibiscus tiliaceus* (Fig.1.11)

A large shrub or small tree to 7 m (23 ft); items are covered with a dense velvety pubescence. The large deeply 3 - 5 lobed leaves become simple and narrower toward the apex of the plant. The large 20 cm (8 in) blooms are produced in spring and summer. These blooms are soft rose to pale pink with crimson colouring at the base of the style. The wine-coloured anthers and stigmas combine to produce a bloom of exquisite beauty. Hence the name splendens from the latin adjective splendidus meaning splendid or bright. The flowers are the most delicate pink and crimson and literally cover the plant. A welcome addition to any hibiscus collection, *H. spendens* tolerates cultivation very well and is easily grown in areas sheltered from strong winds. It naturally occurs as a rainforest plant and is found in deep gullies and gorges with rich fertile well drained soil. It does not mind semi-shade and will usually do well when competing with other plants. Being an Australian native it prefers organic fertilizers to chemical ones. *H. splendens* is grown from seed sown in spring or by firm wood cuttings taken in autumn.

1.2.12 *Hibiscus splendens* (Fig.1.12)

A spreading evergreen tree to 9 m (30 ft), which spreads to 16 m (50 ft). The blooms are 8 - 10 cm across and are bright lemon yellow and distinctly veined. At the base of each petal on its inner surface is a large crimson blotch. The flowers are rarely held erect but tend to turn to one side or face downwards. Individual flowers last two days, and on the second day the bloom changes colour to an orange or brownish pink, and finally the corolla falls entirely from the tree. Blooming from early summer to the end of autumn, *H. tiliaceus* is most prolific in mid-summer, particularly after good rains. The fibrous inner bark was one of the commonest raw materials in the manufacture of tapa cloth throughout the Pacific Islands. The wood is used for out riggers of canoes, parts of cart, handles and household implements. It too reported to be used in the West Indies for cabinet work, flooring, paneling and fancy work. In India it is used as fuel. It can also be used in place of walnut for gunstocks. An infusion of the leaves is used as a lotion for ulcers and wounds; the leaves are considered laxative. Flowers are boiled in milk and used as a remedy for ear ache. *H. tiliaceus* requires a warm situation with liberal watering during summer. It is propagated from seed sown in spring. Selected strains may be grafted onto seedling stock to ensure good flowering habits.

1.2.13 *Hibiscus boryanus* (Fig.1.13)

Hibiscus boryanus is an endangered and protected species. It is a small tree up to a height of 8 m, endemic to the Mascarene Islands. Flowers range from orange to red; it prefers locations with year round moisture but not in excessive amount.

Fig.1.1 Flower of *Hibiscus cameronii*

Fig.1.2 Flower of *Hibiscus coccineus*

Fig.1.3 Flower of *Hibiscus glaber*

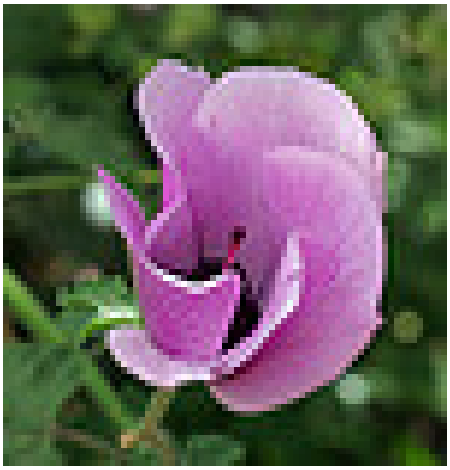


Fig.1.4 Flower of *Hibiscus huegelii*

Fig.1.5 Flower of *Hibiscus insularis*

Fig.1.6.Flower of *Hibiscus manihot*

Fig.1.7 Flower of *Hibiscus moscheutos*

Fig.1.8 Flower of *Hibiscus mutablis*

Fig.1.9 Flower of *Hibiscus trionum*

Fig.1.10 Flower of *Hibiscus sabdariffa*

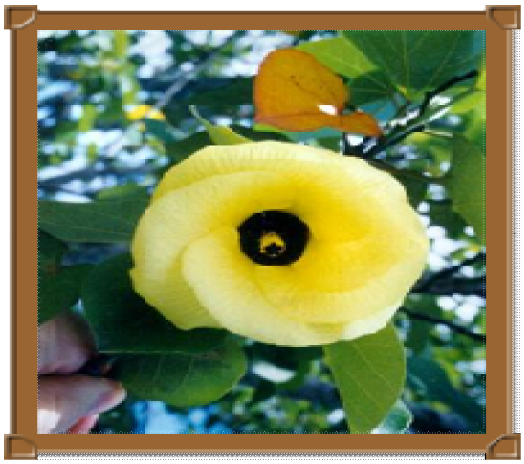


Fig.1.11 Flower of *Hibiscus tiliaceus*

Fig.1.12 Flower of *Hibiscus splendens*

Fig.1.13 Flower of *Hibiscus boryanus*

There are many Hibiscus species used medicinally for a wide variety of ailments. Most commonly medicinally used is *Hibiscus sabdariffa,* known as roselle, but also popular are *Hibiscus rosa-sinensis* (common hibiscus, China rose), *Hibiscus syriacus* (rose of Sharon) and *Hibiscus arnottianus* (Hawaiian hibiscus).

In India, the ayurvedic system has described a large number of medicines based on plants or plant product and the determination of their morphological and pharmacological or pharmacognostical characters can provide a better understanding of their active principles and mode of action. However a large number of tropical plants have not been studied in detail for their chemical constituents, pharmacological properties of the extracts. The present study is based on the phytochemical screening, elemental analysis and antimicrobial activity on flower part of three Hibiscus species (*Hibiscus rosa sinensis, Hibiscus syriacus and Hibiscus arnottianus*).

LITERATURE REVIEW

2. LITERATURE REVIEW

2.1. *Hibiscus rosa sinensis*

History:

Hibiscus has grown for centuries around the rim of the Indian and Pacific oceans. Colonialism in the 18th and 19th centuries helped to give the flower its romantic image. Hibiscus is derived from the Greek word hibiskos, given by the Greek physician Dioscorides in the 1st century to the marshmallow plant, a close relative of the hibiscus. *Hibiscus rosa sinensis*, an old species that was grown as an ornamental flower in china, is believed to have been cultivated there for hundreds and thousands of years.

Description:

Hibiscus rosa sinensis (Family-Malvaceae), is an evergreen flowering shrub native to East Asia. It is widely grown as an ornamental plant throughout the tropics and subtropics. It is a small tree, an ever green shrub, growing to a maximum of 10 m in the wilds. The leaves are 2.5 cm in length and 2-2.5 cm wide. Flowers are pedicillate, actinomorphic, pentamerous, and red in colour and about 3 inches in diameter.

Origin and Distribution:

Hibiscus rosa-sinensis are native to Tropical Asia. A native of South-eastern Asia (China), the plant is commonly found throughout the tropics and as a house plant throughout the world. Most ornamental varieties are hybrids. The present wide range

of cultivars is considered to be a complex of interspecific hybrids, between 8 or more different species originating from the African East Coast and islands in the Indian and Pacific Ocean.

Propagation and Cultivation:

Hibiscus rosa sinensis grows best under moderate temperature and relatively high humid conditions. It thrives best on well drained porous loamy soil. The Plant is usually propagated by cuttings, preferably from mature wood of current year growth. Layering, budding, grafting and air layering can also be successfully applied. Plants propagated by air or ground layering show better growth and flowering. Shoe flower is seriously infected by insects like mites and red spider causing curling of leaves, which stops further growth and flowering.

Parts Used:

Flowers, Roots and Leaves.

Local/ Vernacular name in India: (Jadhav V.M. *et al.*, 2009)

Tamil : Semparutti
Hindi : Jasum
English : Chinese Hibiscus
Gujarat : Jasvua
Sanskrit : Japa
Orissa : Mondaro.
Telugu : Dasanamu

Name in various countries:

Arabic : Angharachindi
Burma : Kaungyan
China : Hong can
French : Rose de china

Fig.2.1 Flowers of *Hibiscus rosa sinensis*

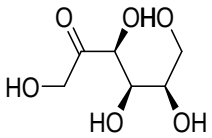
Traditional Medicinal Uses: (Nandan Kumar Jha. *et al*., 2008)

- Decoction of dried flowers was taken orally for abortion. Hot water extract was taken orally as an anti-fertility agent. For this the flowers with their sexual parts were taken orally by the female concerned.
- Flowers and leaves were taken orally for constipation and painful bowel motion.
- The flowers and leaves were churned into a mucilaginous juice with water and filtered. About half a cup of the filtrate was taken by mouth every day before going to bed act as good laxative.
- A decoction of root is used for venereal diseases and fevers.
- Staminal column is diuretic used for Kidney trouble.
- Buds are used in treatment of vaginal and uterine discharges.
- Leaves and flowers are good for healing ulcers and for promoting growth and colour of hair.
- Hot water extract of flowers is taken orally for menorrhagia, bronchitis, as an emnenagogue for treatment of menarche. Flower decoction alone with "jaggary" is drunk, and as a contraceptive in Ayurvedic medicine.

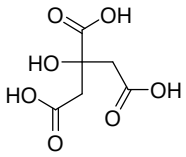
Phytochemistry:

Analysis of the edible part of flowers of *Hibiscus rosa sinensis* gave the following values ; moisture 89.8; nitrogen 0.064, fat 0.36, crude fibre 1.56 %, calcium 4.04, phosphorus 26.68, iron 1.69 mg / 100gm . Flavones from flowers are quercetin-3, 5-diglucoside, quercetin-3, 7- diglucoside and cyanidin-3, 5-diglucoside. The flowers contains vitamins such as thiamine [0.031mg %], riboflavin [0.048 mg %], niacin [0.61 mg %] and ascorbic acid [4.16 mg %], apigenidin, citric acid, fructose, glucose, oxalic acid, pelargonidin, quercetin are also present

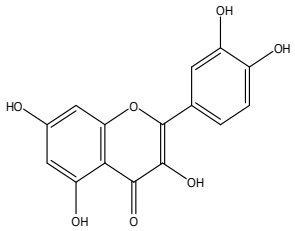
(Jadhav V.M. *et al.*, **2009**).The GC-MS analysis of ethanolic extract of *Hibiscus rosa sinensis* showed the presence twelve compounds in which the major phytochemicals are hexadeconoic acid, hexanedioic acid and squalene (Anusha Bhaskar. *et al*., **2011**).



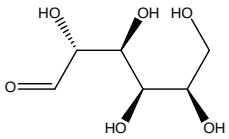
Fructose



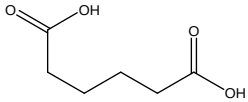
Citric acid



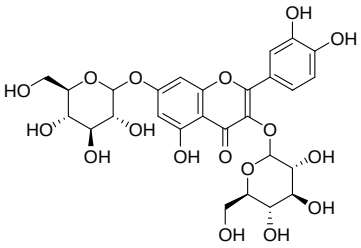
Quercetin



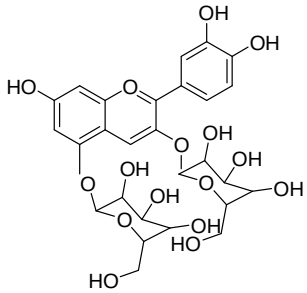
Glucose



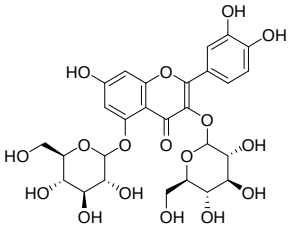
Hexanedioic acid



Quercetin-3, 7 diglucoside



Cyanidin-3, 5-diglucoside



Quercetin-3, 5 diglucoside

Fig.2.2 Compounds isolated from flower of *Hibiscus rosa sinensis*

Pharmacological activities:

1. **Antifertility Activity:**

Kholkute S.D. *et al.*, (1977) studied the postcoital anti fertility properties of benzene hot extracts of *Hibiscus rosa sinensis* flowers, leaves and stem barks, collected during the winter, spring, rainy and summer seasons were investigated in female rats. Only extracts from the flower of the plant were 100% effective in preventing pregnancy. Those flowers collected during the winter showed the greatest potency, followed by those collected in the spring, rainy season and summer in decreasing order.

2. **Abortifacient Activity:**

Jadhav V.M. *et al.*, (2009) performed abortifacient activity of water insoluble and ether soluble fractions of a total benzene extract of dried flowers, administered by gastric intubation to rats at a dose of 186.0 mg / kg were active. Ether soluble and water insoluble fractions of a total benzene extract, at a dose of 73.0 mg /kg, were also active.

3. **Anticonvulsive Activity:**

Kasture V.S. *et al.*, (2000) evaluated anticonvulsant activity of leaves of *Albizzia lebbeck* and flowers of *Hibiscus rosa sinensis* and the petroleum ether extract of *Butea monosperma*. The bioassay guided fractionation indicated that the anticonvulsant activity lies in the methanolic fraction of chloroform soluble part of ethanolic extract of the leaves of *A. lebbeck*, acetone soluble part of ethanolic extract of *Hibiscus rosa sinensis* flower and acetone soluble part of petroleum ether extract of *B. monosperma* flowers. The fraction protected animals from maximum electroshock, electrical kindling and pentylenetetrazole induced convulsions in mice. The fractions also

inhibited convulsions induced by lithium-pilocarpine and electrical kindling. However, they failed to protect animals from strychnine induced convulsions. The fractions antagonized the behavioral effects of D-amphetamine and potentiated the phenobarbitone induced sleep. The fractions raised brain contents of gamma-aminobutyric acid (GABA) and serotonin. These fractions were found to be anxiogenic and general depressant of central nervous system.

4. Antiestrogenic Activity:

Prakash A.O. (1979) performed the antiestrogenic activity of total alcoholic and benzene extracts of air dried and powdered *Hibiscus rosa sinensis* flower in bilaterally ovariectomized immature female albino rats. Uterine weight gain was stimulated by pretreatment with estrone. Administration of 150, 200 and 250 mg/kg of the total alcohol extract significantly decreased the estrone induced increase in uterine weight. Oral administration of 250 mg/kg of the benzene extract had a similar effect, but was more potent than the alcohol extract. The activity of the extract was dose-dependent.

5. Antiovulatory Activity:

Murthy D.R. *et al.*, (1997) studied that the benzene extract of *Hibiscus rosa sinensis* flower, administered intraperitoneally to adult mice at doses of 125 and 250 mg / kg body weight to adult mice and resulted in an irregular estrous cycle with prolonged estrus and metestrus. An increase in the atretic follicles and the absence of corpora lutea indicate the antiovulatory effect of the extract. The extract also showed estrogenic activity in immature mice by early of the vagina, premature cornification of the vaginal epithelium and an increase in uterine weight. Therefore the ovulatory effect may be an

increase in uterine weight. Therefore the antiovulatory effect may be due to an imbalance in the hormonal environment, as there may be an increase in the endogenous secretion of estrogen by atretic follicles, and also to the estrogenicity of the flower extract.

6. Hair Growth Activity:

Adhiraj N. *et al.*, (2003) evaluated the petroleum ether extract of leaves and flowers of *Hibiscus rosa sinensis* for its potential on hair growth by *in vivo* and in vitro methods. *In vivo*, 1% extract of leaves and flowers in liquid paraffin was applied topically over the shaved skin of albino rats and monitored and assessed for 30 days. The length of hair and the different cyclic phases of hair follicles like anagen and telogen phases were determined at different time periods. *In vitro*, the hair follicles from albino rat neonates were isolated and cultured in DMEM supplemented with 0.01 mg/ml petroleum ether extract of leaves and flowers. From the study it is concluded that the leaf extract exhibits more potency on hair growth when compared to flower extract.

7. Anti-diabetic activity:

Venkatesh S. *et al.*, (2008) performed the anti-diabetic activity of ethanol extract of flowers of *Hibiscus rosa sinensis* at doses of 250 mg/kg and 500 mg/kg significantly reduced the blood glucose level in both acute and sub acute treatments.

8. Antioxidant activity:

Yamasaki H. *et al.*, (1996) performed antioxidant activity in which the red anthocyanin prepared from petals of *Hibiscus rosa sinensis* was photobleached in EDTA riboflavin system. The rate of bleaching monitored at

565 nm depended on the light intensity and EDTA concentrations. Anaerobic conditions or addition of superoxide dismutase prevented the bleaching of anthocyanin, whereas mannitol and catalase did not. The results indicate that anthocyanin is bleached by the nonenzymatic reaction with the superoxide radical and suggest that the pigment can function as an antioxidant.

9. Cardioprotective effect:

Gauthaman K.K. *et al.*, (2006) evaluated cardio protective effect of the *Hibiscus rosa sinensis* flower in an oxidative stress model of myocardial ischemic reperfusion injury in rat. Dried pulverized flower of *Hibiscus rosa sinensis* was administered orally to wistar albino rats (150-200 g) in three different doses (125, 250 and 500 mg/kg) for 4 weeks. Thereafter, rats were sacrificed either for the determination of baseline changes in cardiac endogenous antioxidants or the hearts were subjected to isoproterenol induced myocardial necrosis. There was significant increase in the baseline contents of thiobarbituric acid reactive substances with both doses of *Hibiscus rosa sinensis*. It may be concluded that flower of *Hibiscus rosa sinensis* (250 mg/kg) augments endogenous antioxidant compounds of rat heart and also prevents the myocardium from isoproterenol induced myocardial injury.

10. Cytotoxicity activity:

Ozmen. *et al.*, (2010) showed significant antimitotic activity of flower decoction of *Hibiscus rosa sinensis*. It can be suggested that *Hibiscus rosa-sinensis* flowers contains antimitotic constituents which stop the cell division in anywhere of the cell cycle.

11. Embryotoxic effect:

Sign M.P. *et al.*, (1982) performed embryotoxic effect of benzene extract of dried flower of *Hibiscus rosa sinensis*, administered by gastric intubation to pregnant rats at doses (100, 150, 186 mg/kg) for 1-10 days were active. The ether soluble and water insoluble fractions of the total benzene extracts were also active.

12. Hypotensive activity:

Siddiqui A.A. *et al.*, (2006) evaluated phytochemical and pharmacological investigation of flowers of *Hibiscus rosa sinensis*. The flowers of *Hibiscus rosa sinensis* were extracted with three solvents. The extracts were dried and studied for their hypotensive activity. Among the crude extract, hydroalcoholic extract was found to show most significant activity with reference to the standard minoxidil.

13. Wound healing activity:

Shivananda Nayak B. *et al.*, (2007) studied the effect of *Hibiscus rosa sinensis* on wound healing activity: a preclinical study in a Sprague Dawley rat. The wound healing activity of the ethanol extract of *Hibiscus rosa sinensis* flower was determined in rats, using excision, incision, and dead space wound models. The animals were randomly divided into 2 groups of 6 in each in all the models. Test group animals, in each model were treated with the ethanol extract of *Hibiscus rosa sinensis* and the control group animal were maintained with plain drinking water. Healing was assessed by the rate of wound contraction, period of epithelialisation, tensile strength, granulation tissue weight and hydroxyproline content. The antimicrobial activity of the

flower extracts against selected microorganisms that infect the wounds was also assessed.

14. Antibacterial activity:

Vimalin Hena V. (2010) performed antibacterial activity of the flowers of *Hibiscus rosa sinensis* against two gram positive bacteria (*Staphylococcus aureus* and *bacillus subtillis*) and two gram negative bacteria (*Escherichia coli* and *Pseudomonas aeruginosa*).

15. Anti-inflammatory activity:

Singh N. *et al.*, 1978 evaluated anti-inflammatory activity of ethanolic extract of dried leaves of *Hibiscus rosa sinensis* by carrageenan induced paw edema models. The extracts were administered intraperitoneally to rats at a dose of 100 mg/kg and were found active.

16. Antipyretic and antispasmodic activity:

Bhakuni O.S. *et al.*, (1969) investigated the antipyretic activity of extracts of dried leaves of *Hibiscus rosa sinensis.* The ethanolic extract was administered intraperitoneally to rats at a dose 100 mg/kg was active against brewer's yeast induced pyrexia. The ethanol/water (1:1) extact of aerial parts, administered intraperitoneally to the mice at 500 mg/kg was found to be active. The aqueous ethanolic extract of the aerial parts of *Hibiscus rosa sinensis* was also studied for spasmolytic effect to explain its traditional use in constipation and diarrhea.

17. Antifungal activity:

Renu. (1983) evaluated antifungal activity of ethanol/water (50%) extract of the dried leaves of *Hibiscus rosa sinensis* was active against *Rhizoctonia solani*.

18. Antiviral activity:

Van Den Berghe. *et al*., (1978) screened the antiviral activity on ethanolic extract of freeze-dried plant of *Hibiscus rosa sinensis*. The cell culture at variable concentration was equivocal on Coxsakie B_2 virus, measles virus and polio virus I and inactive on adenovirus, Herpes virus type I and Semlicki-forest virus vs plaque inhibition.

19. Acid phosphatase stimulation:

Prakash A.O. (1979) reported the effect of benzene and ethanol/water (1:1) extracts of *Hibiscus rosa sinensis* flowers, on the estrogen dependent enzyme (acid and alkanine phosphatase) activity of rat uterus were found active at both 150 mg/kg and 300 mg/kg.

20. Ameliorative potential:

Sharma S. *et al*., (2004) conducted the ameliorative potential of *Hibiscus rosa sinensis* in mice skin. The extract exerts a protective effective against the tumour promotion stage of cancer development induced by topical application of benzoyl peroxide followed by ultraviolet radiation.

21. Aphrodisiac activity:

Olagbende Dada S.O. *et al*., (2007) employed aphrodisiac activity of cold aqueous extract of *Hibiscus rosa sinensis* leaves. The extract showed significant anabolic effect on the tested rats.

2.2. *Hibiscus syriacus*

History:

Hibiscus syriacus, a hardy hibiscus shrub, has been a garden shrub in Korea. It was grown in Europe from the 16th century, by the end of the 17th century some knew it to be hardy: Gibson, describing Lord Arlington's London house noted six large earthen pots coddling the "tree hollyhock", as he called it, "that grows well enough in the ground". By the 18th century the shrub was common in English gardens and in the American colonies, known as *Althea frutex* and "Syrian ketmia".

Description:

Hibiscus syriacus (Family-Malvaceae), is grown as a common ornamental plant with many varieties. Shrubs usually under 8' tall in cultivation but may be 15' tree in tropics. Ovoid glossy green separate leaves 6" long. Flowers in upper leaf are solitary, with petals 2-5" long and bright red, or at times pink, purple, orange, yellow, or white. Cultivars may be single, double and colored red, yellow, white or orange. Fruit is a 5-celled capsule each with 3 seeds. Grows primarily in the tropics and was originally cultivated in Asia.

Origin and Distribution:

Hibiscus syriacus is distributed in Asia including China, Northern India, Japan, Korea and also in Middle East, Europe and North America where it was found before 1600. For about 100 days from early July to late October it is flower season and each day it blooms. During that time, a single plant can produce about 2000 to 3000 flowers. Because it blooms a lot of flowers it has strong rate of survival and also

when it is transplanted to other area or cut off it can recover easily. Because of that it represents long and healthy life in Korea.

Propagation and Cultivation:

Hibiscus syriacus is fairly easily propagated from either seeds, with variable results, or by layering or cuttings, cloning the original.

Parts Used:

Flowers, bark and roots.

Vernacular names:

English : Rose of Sharon.

Spanish : Rosa de siria.

Korean : Moo goong hwa.

French : Ketmie de jardins.

Danish : Havehibiscus.

Japanese : Mukuge.

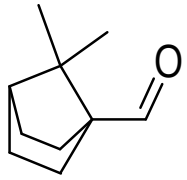
Traditional Medicinal Uses:

- Infusion of dried flowers used as a diuretic. Also used for itches and other skin ailments.
- It is used as analgesic, anti-inflammatory to treat trauma.
- The flowers also used for anthelmintic, urethritis, headache, tooth ache, ear ache, asthma, boils, burns, cough, fever, laxative, litholytic, menstrual irregularity and prostate disorders.
- Decoction of flowers used for dizziness and bloody stools.
- In Indo-China, used for dysentery.
- Flowers sometimes used as substitute for tea.
- Blue dye obtained from the flowers.

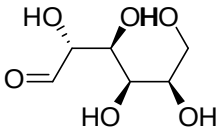
Fig.2.3 Flower of *Hibiscus syriacus*

Phytochemistry:

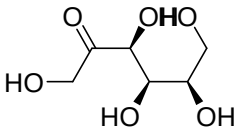
The flowers of *Hibiscus syriacus* were found to contain flavonoids such as apigenidine, palargonidine, cyanidine, quercitine, crisantemin and anthocyanine. kaempherol, camphoral, citric,oxalic acids, and tartaric acid were also present. The Juice of flower contains glycosides, triterpenoids, lipids, terpines, beta-sitosterol, teraxeril, cyanidic glycosides. Miscellaneous substances such as sucrose, fructose glucose were also present (Lana Dvorkin. *et al.*, **2002**).



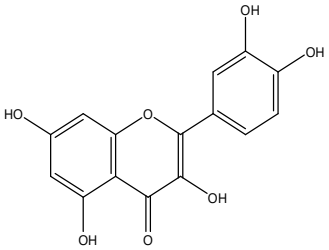
Camphorol



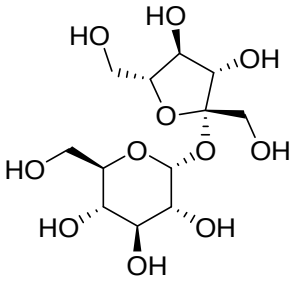
Glucose



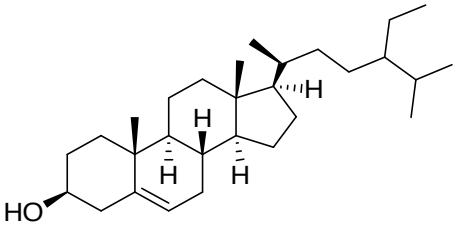
Fructose



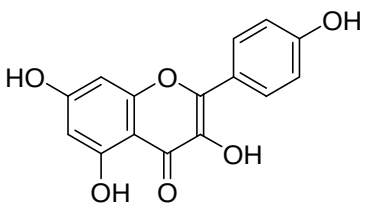
Quercetin



Sucrose



Beta-sitosterol



Kaempferol

Fig.2.4 Compounds isolated from flower of *Hibiscus syriacus*

Pharmacological activities:

1. **Antioxidant:**

Kwon S.W. *et al.*, (2003) performed antioxidant properties of heat-treated *Hibiscus syriacus*. The antioxidant properties of heat-treated *Hibiscus syriacus* was investigated using DPPH test. The stems and the roots of *Hibiscus syriacus* were examined, respectively. As a result, the extracts of heat-treated *Hibiscus syriacus* at 100º C for 24 h were more effective than those of non-treated *Hibiscus syriacus* in reducing the stable free radical 1, 1-Dipheny 1-2-picrylhydrazyl (DPPH).

2. **Naphthalenes / *C*ytotoxicity / Anti-Lipid Peroxidantion:**

Yoo I.D. *et al.*, (1998) isolated three naphthalenes from root bark of *Hibiscus syriacus*. Three new naphthalenes, designated as syriacusins A-C, were isolated from the root bark of *Hibiscus syriacus*. These compounds were identified as 2, 7-dihydroxy-6-methyl-8-methoxy-1-Naphthalene carbaldehyde, 2-hydroxy-6- hydroxyl methyl-7, 8-dimethoxy-1-naphthalenecarbaldehyde,1-carboxy-2,8-dihydroxy-6-methyl,7-meoxy naphthale necarbolactone , respectively, on the basis of various spectral studies. The compounds inhibited lipid peroxidation with IC50s of 0.54, 5.90 and 1.02 micrograms ml-1, respectively. The first compound also showed cytotoxicity against some human cancer cell lines with an ED50 of 1.5-2.4 micrograms ml-1.

3. Anthocyanidin Malonylglucosides:

Jong Hwa Kim. *et al*., (1989) studied that methanolic formic acid extract of petals yielded 3-O-malonylglucosides of delphinidin, cyanidin, pentunidin, pelargonidin, peonidin and malvidin.

4. Triterpene Esters / Lipid Peroxidation Inhibition / Cytotoxicity:

Yun B.S. *et al*., (1999) isolated two bioactive pentacyclic triterpene esters from the root bark of *Hibiscus syriacus*. Two new triterpene caffeates have been isolated from the root bark of *Hibiscus syriacus*. Their structures were established through various spectral studies as 3beta, 23, 28-trihydroxy-12-oleanene 23-caffeate and 3beta, 23, 28-trihydroxy-12-oleanene 3beta-caffeate. Both the compounds showed lipid peroxidation inhibitory activity and significant cytotoxicity against a panel of human cancer cell lines.

5. Coumarins / MAO Inhibitory Activity:

Yun B.S. *et al*., (2001) performed Coumarins with monoamine oxidase inhibitory activity and antioxidative coumarino-lignans from *Hibiscus syriacus*. A previously undescribed coumarin and a new coumarino-lignan, together with the known compounds scopoletin and cleomiscosins A, C, and D, have been isolated from the root bark of *Hibiscus syriacus*. The new coumarino-lignan and cleomiscosin C showed lipid peroxidation inhibitor activity.

2.3. *Hibiscus arnottianus*

History:

Hibiscus arnottianus subsp. *immaculatus* is endemic to East Molokai, and known only from three populations at Olokui above Waieheu, Papalaua, and Wailau. Historically, it was also known from Kalae on Molokai. While it was reported from Waihanau Valley, in the Kalaupapa National Historical Park on Molokai, this report has never been confirmed with voucher specimens (Hawaii Biodiversity and Mapping Program 2009). West of Papalaua Valley, above Kikipua Point, at 427 to 457 meters (1,400 to 1,500 feet) elevation six trees were seen in 1989. In 1990, 20 or more trees were seen in a forested area above sea cliffs in Wailau Valley on a ridge leading to Olokui at 480 meters.

Description:

Hibiscus arnottianus subsp. Immaculatus (Family-Malvaceae), is a shrub or small tree generally 8 meters in height, though individuals may reach 10 meters tall. The leaves are 4 to 10 centimeters long and often have red veins and stems. The faintly fragrant flowers have white petals 8 to 11 cm long, 2.5 to 3.5 cm wide, with the calyx being 2.5 to 3 centimeters long. Anthers are arranged along the upper third of the white staminal column 10 to 14 cm long. This subspecies is distinguished from the other native Hawaiian members of its genus by its white petals and white staminal column.

Origin and Distribution:

Hibiscus arnottianus is an endemic Hawaiian plant with one endangered subspecies (*Hibiscus arnottianus* ssp. *immaculatus)*, which is the rarest of the 11 Hibiscus taxa native to Hawaii. The species is mostly found in mesic to wet forests, at

300 to 800 meters elevations of the Ko'olau and Wai'anae mountain ranges of O'ahu and Wailau Valley on Moloka'i.

Propagation and Cultivation:

Propagation by Seeds:

The seeds of *Hibiscus arnottianus* are contained in papery 1/2 to 1 inch capsules which are tanor brown colored when ripe. The capsules split open when mature and the seeds fall to the ground. The fuzzy seeds are 1/8 inch long and yellowish brown. *Hibiscus arnottianus* is easy to grow from fresh seed, but it hybridizes easily and the seedlings may differ from the parent plant. Using a paint brush, transfer pollen to the stigma of the flower and then enclose the flower in a bag until the seed capsule ripens.

It was recommends hand pollination in the early morning. Pick the capsules just as they turn tan and before they open up. Place them in a container such as a paper bag to dry. As the capsule dries, the seeds will fall out or they can be removed manually. Soak the seeds in water overnight and plant the ones that sink. Use a well-drained sterile potting mix such as 2 parts potting soil and 1 part perlite and keep the mix moist.

Propagation by Cuttings:

Hibiscus arnottianus grow easily from semi-hardwood cuttings. Cuttings 4 to 6 inches long and less than 1/2 inch in diameter should be made from healthy branches without flower buds. Usually recommends terminal or sub-terminal cuttings. Reduce transpiration by removing half of each leaf above the rooting medium and use a medium strength rooting hormone. Keep the cuttings in a humid environment and the rooting medium moist.

Propagation by Air Layers:

Hibiscus arnottianus can be air layered. Use standard air layer technique on a branch that is about 1 inch in diameter. Select a branch that is growing upright and making the air layer between 1 and 2 feet from the tip of the branch.

To start a plant by air layering, remove the bark and cambium from a 1 inch wide ring of bark. Apply a rooting hormone to the cut surface and cover this with a layer of damp sphagnum moss. Wrap the moss in plastic being sure to secure the ends where it wraps around the branch. The air layer should be ready to remove from the parent plant in 3 to 5 months. It was suggested that suggests that root systems from air-layered plants are not as vigorous as those produced by other techniques.

Vernacular names:

English : Hawaiin hibiscus.

Spanish : Hibisco de Arnott.

Scientific classification:

Kingdom : Plantae-Plants

Subkingdom : Tracheobionta-Vascular plants

Super division : Spermatophyta-Seed plants

Division : Magnoliophyta-Flowering plants

Class : Magnoliopsida-Dicotyledons

Subclass : Dilleniidae

Order : Malvales

Family : Malvaceae-Mallow Family

Genus : Hibiscus L. - Rosemallow

Species : *Hibiscus arnottianus* Gray - White Rosemallow

Subspecies : *Hibiscus arnottianus* A. Gray ssp. *immaculatus*

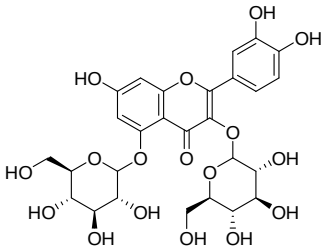
Fig.2.5 Flowers of *Hibiscus arnottianus*

Traditional Medicinal Uses:

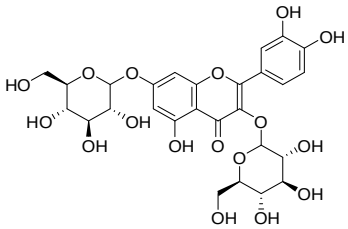
- The flower buds of the Koki'o ke'oke'o were used as a mild laxative by the early Hawaiians.

Phytochemistry:

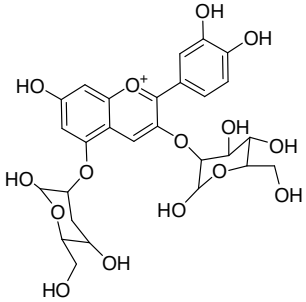
The flowers of *Hibiscus arnottianus* contains flavones such as quercetin-3, 5-diglucoside, quercetin-3, 7- diglucoside, cyanidin-3,5-diglucoside and kaempferol-3-xylosyl glucoside. (Jadhav V.M. *et al.*, **2009**)



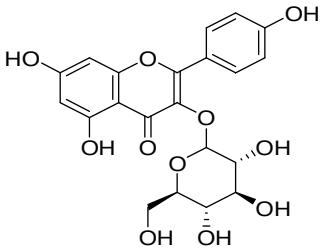
Quercetin-3, 5 diglucoside



Quercetin-3, 7 diglucoside



Cyanidin-3, 5-diglucoside



kaempferol-3-xylosyl glucoside

Fig.2.6 Compounds isolated from *Hibiscus arnottianus*

Pharmacological studies: Not yet reported

AIM

&

PLAN OF WORK

3. AIM AND PLAN OF WORK

Aim:

The present study was designed to find out the chemical composition of the flower part of *Hibiscus rosa sinensis, Hibiscus syriacus* and *Hibiscus arnottianus* by using novel instrumentation techniques as a part of their standardization and compared their antimicrobial activity.

Plan of work:

1. Collection and authentication of flower part of Hibiscus species.
2. Extraction of the flower of Hibiscus species by cold extraction method.
3. Qualitative screening of phytochemicals and vitamins in methanolic flower extracts of Hibiscus species.
4. Quantitative analysis of phytochemicals and vitamins in flower of Hibiscus species.
5. Preparation of total ash.
6. Qualitative screening of inorganic elements in flower of Hibiscus species.
7. Estimation of inorganic elements (Ca, P, Fe) in flower of Hibiscus species by AAS method.
8. Proximate analysis in flower of Hibiscus species.
9. TLC study in methanolic flower extracts of Hibiscus species.
10. UV and FTIR fingerprinting in methanolic flower extracts of Hibiscus species.
11. HPTLC Finger printings in methanolic flower extracts of Hibiscus species.
12. Analysis of chemical composition in methanolic flower extracts of Hibiscus species by Gas Chromatography-Mass Spectroscopic method.
13. Antimicrobial activity in methanolic flower extracts of Hibiscus species.

EXPERIMENTAL

4. EXPERIMENTAL

4.1. Materials:

4.1.1. Plant:

The three species of **Hibiscus flower** (Family: *Malvaceae*) were collected from Kundrathur, Chennai in the month of July 2011 as follows:

1. *Hibiscus rosa sinensis*(red)
2. *Hibiscus syriacus*(pink)
3. *Hibiscus arnottianus*(white)

They were authenticated by Prof. P. Jayaraman, Taxonomist, Plant Anatomy Research Centre (PARC), Chennai.

4.1.2. Chemicals:

Table.4.1. List of Chemicals/Drugs

S.No	Chemicals	Manufacturer
1.	Ammonium molybdate	Qualigens Fine Chemicals, Mumbai
2.	Ammonia	Analytical Reagents, Mumbai
3.	Ascorbic acid	Loba Chemie Pvt. Ltd, Mumbai
4.	Diethyl ether	Loba Chemie Pvt. Ltd, Mumbai
5.	Dichloromethane	Analytical Reagents, Mumbai
6.	EDTA(ethylenediamine tetra acetic acid)	Qualigens Fine Chemicals, Mumbai
7.	Ethanol	Loba Chemie Pvt. Ltd, Mumbai
8.	Folin-coicalteau	Qualigens Fine Chemicals, Mumbai
9.	Hexane	Analytical Reagents, Mumbai
10.	Methanol	Qualigens Fine Chemicals, Mumbai
11.	Niacin	Loba Chemie Pvt. Ltd, Mumbai
12.	Oxalic acid	Analytical Reagents, Mumbai

S.No	Chemicals	Manufacturer
13.	Potassium dichromate	Loba Chemie Pvt. Ltd, Mumbai
14.	Potassium cyanide	Qualigens Fine Chemicals, Mumbai
15.	Riboflavin	Loba Chemie Pvt. Ltd, Mumbai
16.	Sulphuric acid	Loba Chemie Pvt. Ltd, Mumbai
17.	Trichloroacetate	Loba Chemie Pvt. Ltd, Mumbai
18.	Thiamine	Loba Chemie Pvt. Ltd, Mumbai
19.	Ketaconazole and ciprofloxacin disc	Hi-Media Pvt.Ltd, Mumbai
20.	Nutrient agar medium, sabouraud's dextrose agar medium	Hi-Media Pvt.Ltd, Mumbai

4.1.3. Instruments:

Table.4.2 List of Equipments

S.No	Instruments	Manufacturer
1.	Electronic weighing balance	SHIMADZU AUX 220, Chennai.
2.	Muffle furnace	Dolphin Industries Ltd., Mumbai.
3.	Rotary shaker	REMI Equipments, Chennai.
5.	HPTLC	CAMAG Linomate IV, Switzerland.
6.	GC-MS	PerkinElmer Clarus 500, Japan.
7.	FT-IR	Perkin-Elmer, Mumbai.
8.	UV-Visible spectrometer	Shimadzu-1700, Japan.
9.	Atomic absorption spectrophotometer	Perkin Elmer Model 403, Norwalk, CT, USA.

4.1.4. Microorganisms:

A gram positive bacteria (*S. aureus*) , gram negative *(E.coli)*and antifungal organism (*A.niger*) were collected from Microbial Resource Division, King's Institute of Preventive Medicine, Guindy, Chennai. The agar medium and sabouraud's dextrose medium was purchased from HI-Media Laboratories Ltd., Mumbai, India.

4.2. Extraction of flower of Hibiscus species by cold extraction method: (Sangeetha Arullapan. *et al*., **2009**).

The dried, powdered flowers of Hibiscus species were extracted using petroleum ether, ethyl acetate and methanol by cold extraction.

10 g of air dried powder of the flowers of Hibiscus species were weighed and placed in 100 ml of organic solvent (petroleum ether, ethyl acetate and methanol) in a conical flask and then kept in a rotary shaker at 190-220 rpm for 24 h. It was filtered with the help of muslin cloth and centrifuged at 5000Xg for 15 min. The supernatant was collected and the solvent was evaporated at 40° C to make the final volume of one-fourth of the original volume, giving a concentration of 40 mg/0.1 ml. The small volume was later dried and the gummy extract was kept in the freezer (–70° C).

$$\% \text{ Yield of extract} = \frac{\text{Weight of extract}}{\text{Weight of powder taken}} \times 100$$

4.3. Qualitative screening of phytochemicals and vitamins in methanolic flower extracts of Hibiscus species:

The methanolic flower extracts of Hibiscus species were subjected to qualitative test using standard procedures to identify various constituents. (Ghosh A.K. *et al*., **2010**)

Phytochemicals:

4.3.1. Test for carbohydrate:

- **Molisch's test:** To the 0.5 ml of extract, added few drops of alcoholic alpha napthol and 0.2 ml of concentrated sulfuric acid slowly through the sides of the test tube, a purple to violet color ring appears at the junction.

- **Benedict's test:** The extract was treated with few drops of Benedict's reagent (alkaline solution containing cupric citrate complex) and boiled on water bath, reddish brown precipitate forms if reducing sugars are present.

4.3.2 Test for Proteins & Amino Acids:

- **Millon's test:** 0.5 ml of extract was treated with 2 ml of Millon's reagent (Mercuric nitrate in nitric acid containing traces of nitrous acid), white precipitate appears, which turns red upon gentle heating.
- **Ninhydrin test:** Few drops of extract was boiled with 0.2% solution of Ninhydrin (Indane 1, 2, 3 trione hydrate), violet color appears indicated the presence of Amino acids and Proteins.

4.3.3 Test for Alkaloids:

- **Mayer's test:** Alkaloids gives cream color precipitate with Mayer's reagent [Potassium mercuric iodide solution].
- **Dragendroff's test:** Alkaloids gives reddish brown precipitate with Dragendroff's reagent [Potassium bismuth iodide solution].
- **Wagner's test:** Alkaloids gives a reddish brown precipitate with Wagner's reagent [Solution of iodine in potassium iodide].
- **Hager's test:** Alkaloids gives yellow color precipitate with Hager's reagent [Saturated solution of Picric acid].
- **Tannic acid test:** Alkaloids gives buff color precipitate with 10% Tannic acid solution.

4.3.4 Test for Glycosides:

The extract was hydrolyzed with mineral acid and then tested for the glycone and aglycone moieties.

- **Raymond's test:** Test extract when treated with dinitro-benzene in hot methanolic alkali, gives violet colour.
- **Legal's test:** The extract was treated with pyridine and alkaline sodium nitroprusside solution was added, blood red color appears.
- **Bromine water test:** Test extract when treated with bromine water gives yellow precipitate.

A. Test for Saponin Glycosides:

Froth Test:

Place 1ml of extract in water in a semi-micro tube and shaken well and noted for a stable froth.

B. Test for Anthraquinone Glycosides:

- **Borntrager's test:** The extract was boiled with 1ml of dil. sulphuric acid in a test tube for 5 min (anthracene glycosides are hydrolyzed to aglycone and sugars by boiling with acids) centrifuge while hot (if centrifuged hot, the plant material can be removed while anthracene aglycones are still sufficiently soluble in hot water, they are however insoluble in cold water), pipetted out the filtrate and was cooled and shaked with an equal volume of dichloromethane (the aglycones will dissolve preferably in dichloromethane). The lower dichloromethane layer was separated and was shaked with half its volume with dilute ammonia. A rose pink to red color is produced in the ammonical layer (aglycones based on anthroquinones gives red color in the presence of alkali).

C. Test for Cardiac Glycosides:

- **Keller killiani test [Test for Deoxy sugars]:** The extract was added with 0.4 ml of glacial acetic acid containing a trace amount of ferric chloride. It was

transferred to a small test tube and then 0.5 ml of conc. sulphuric acid was added carefully by the side of the test tube, blue colour appears in the acetic acid layer.

4.3.5. Test for Flavonoids:

- **Shinoda test (Magnesium Hydrochloride reduction test):** To the extract, few fragments of magnesium ribbon was added followed by conc. hydrochloric acid drop wise, pink scarlet, crimson red or occasionally green to blue color appears after few minutes.
- **Zinc Hydrochloride reduction test:** To the extract a mixture of zinc dust and conc. hydrochloric acid was added. It gives red color after few minutes.
- **Alkaline reagent test:** To the extract few drops of sodium hydroxide solution was added, formation of an intense yellow color, which turns to colorless on addition of few drops of dilute acid, indicates presence of flavonoids.

4.3.6. Test for phenolic compounds and tannins:

- **Test for phenolic compounds:** To the extract, dilute ferric chloride was added. Blue colour was formed.
- **Test for Tannins:**

(a)To the extract, 1% of gelatin and 10% sodium chloride were added a white precipitate formed.

(b)The presence of white precipitate in the test solution, when treated with lead acetate solution indicates presence of tannins.

4.3.7. Test for Sterols & Triterpenoids:

- **Libermann- Buchard test:** To the extract few drops of acetic anhydride was added, boiled and cooled then conc. sulfuric acid was added from the sides of

the test tube, shows a brown ring at the junction of two layers and the upper layer turns green which shows the presence of steroids and formation of deep red color indicates the presence of triterpenoids.

- **Salkowski test:** To the extract in Chloroform was added with few drops of conc. Sulfuric acid, shaken and allowed standing for some time, red color appears at the lower layer indicated the presence of Steroids and formation of yellow colored lower layer indicates the presence of triterpenoids.

4.3.8. Test for Fats & Fixed Oils:

- **Stain test:** The small quantity of extract was pressed between two filter papers; the stain on a filter paper indicates the presence of fixed oils.
- **Saponification test:** To the extract a few drops of 0.5 N of alcoholic potassium hydroxide was added along with a drop of phenolphthalein and was heated on a water bath for 1-2 h. The formation of soap or partial neutralization of alkali indicates the presence of fixed oils and fats.

Vitamins: (Indian pharmacopoeia., **2007**)

4.3.9. Test for Thiamine:

- To the extract, mercuric chloride was added. A white precipitate was produced.
- To the extract, iodine solution was added. A brown precipitate was produced.

4.3.10. Test for Niacin:

- The extract was dissolved in water, and then 0.1 M sodium hydroxide was added. A blue precipitate was produced.
- To 0.5 ml of extract, few drops of cyanogens bromide and aniline were added. A golden yellow colour was produced.

4.3.11. Test for Ascorbic acid:

To the 0.1 ml of extract, few drops of water were added followed by sodium bicarbonate and ferrous sulphate. A deep violet color was produced which disappears on addition of sulphuric acid.

4.3.12. Test for Riboflavin:

To the extract, hydrochloric acid was added which causes disappearance of colour.

4.4. Quantitative analysis of phytochemicals and vitamins in methanolic flower extracts of Hibiscus species:

4.4.1. Phytochemicals:

- **Determination of total phenols:** To determine the total phenols, 5 g of the plant sample was weighed into a 250 ml titration flask and 100 ml n-hexane was added twice for 4 h each; the filtrates were discarded for fat free sample preparation. Then, 50 ml diethyl ether was added twice, was heated for 15 min each, was cooled up to room temperature and was filtered into a separating funnel. About 50 ml of the 10% sodium hydroxide solution was added twice and shaken well each time to separate the aqueous layer from the organic layer. It was washed three times with 25 ml de-ionized water. The total aqueous layer was acidified up to pH 4.0 by adding 10% hydrochloric acid solution and 50 ml dichloro methane (DCM) twice to acidify the aqueous layer in the separating flask. Consequently, the organic layer was collected, dried and then weighed. (Iqbal Hussain. *et al.*, **2011**)
- **Determination of total alkaloids**: For alkaloids determination, 5 g of each sample was weighed into a 250 ml beaker, and 200 ml of 20% acetic acid in ethanol was added and was covered to stand for 4 h. This was filtered and the

extract was concentrated using a water bath to evaporate one-quarter of the original volume. The concentrated ammonium solution was added drop-wise to the extract until the precipitation was completed. The entire solution was allowed to settle and the precipitate was collected by filtration, after which it was, weighed. (Obadoni. *et* al., **2001**)

- **Determination of flavonoids:** To determine flavonoids, 5 g of each plant sample was weighed in a 250 ml titration flask, and 100 ml of the 80% aqueous methanol was added at room temperature and shaken for 4 h in an electric shaker. The entire solution was filtered through Whatman filter paper no. 42 (125 mm) and again, this process was repeated. The filtrate as a whole was later transferred into a crucible and evaporated to dryness over a water bath and weighed. (Boham. *et al*., **1994**)
- **Determination of tannins:** 500 mg powdered sample material was transferred to 250 ml conical flask containing 75 ml of distilled water. The contents in the flask were boiled for 30 min, centrifuged for 2000 rpm for 20 min. The supernatant was collected in 100 ml volumetric flask and made up to a known volume. One ml of the sample extract was transferred to a 100 ml volumetric flask containing 75 ml of distilled water. To this 5 ml of Folin-Denis reagent and 10 ml of sodium carbonate solution were added and diluted to 100 ml. It was shaken well and left for 30 min and the absorbance was read at 700 nm against a reagent blank. (Sadasivam S. *et al*., **1992**)
- **Determination of saponins:** For the saponins determination, 5 g of each plant samples was weighed and was dispersed in 100 ml of 20% ethanol. The suspension was heated over a hot water bath for 4 h with continuous stirring at about 55° C. The filtrate and the residue were re-extracted with another

100 ml of 20% ethanol. The combined extracts were reduced to 40 ml over water bath at about 90° C. The concentrate was transferred into a 250 ml separating funnel and 20 ml of diethyl ether was added and shaken vigorously. The aqueous layer was recovered while the ether layer was discarded. The purification process was repeated and about 30 ml of n-butanol was added. The combined n-butanol extracts were washed twice with 10 ml of 5% aqueous sodium chloride. The remaining solution was heated in a water bath. After evaporation, the samples were dried in the oven to a constant weight. The saponins content was calculated in percentage. (Obadoni. *et* al., **2001**)

- **Determination of total carbohydrates:** 100 mg of dried sample powder was homogenized with 5 ml of 80 % ethanol and centrifuged at 2000 rpm for 10 min. Then it was re-extracted with the same solvent and centrifuged again. The supernatant were pooled. To the supernatant, equal volume of petroleum ether was added to remove the chlorophyll pigments using separating funnel. The lower layer was taken as sample. 1 ml of protein free carbohydrate solution was mixed with 4 ml of the anthrone reagent. The reaction mixture was heated for 5 min in a boiling water bath at 100º C with the marble on the top of the test tube to prevent loss of water by evaporation. Suitable reagent blank was prepared. The colour intensity was measured at 620 nm. (Shanthi P. *et al*., **2010**)
- **Determination of total protein content:** 100 mg of sample was homogenized with 5 ml of ice-cold phosphate buffer and centrifuged at 2000 rpm for 5 min. To the supernatant solution, equal volume of 10% ice-cold trichloro acetate was added and incubated for 10 min at 4° C for an hour. The precipitated protein was centrifuged and the pellet was dissolved in 1 ml of 0.1N sodium

hydroxide. 0.5 ml of the protein solution was mixed with 5 ml of alkaline copper reagent. It was shaken well and allowed to stand at room temperature for 10 min. Then, 0.5 ml of folin–ciocalteau reagent was added and the volume was made up to a known quantity using distilled water. Blank was prepared without the sample extract. After 30 min the absorbance of the solution was read at 660 nm. (Lowry O H. *et al.,* **1951**)

4.4.2. Vitamins: (Deepak Koche. **2011**)

- **Thiamine**: 5 g of sample was homogenized in 50 ml ethanolic sodium hydroxide. Its 10 ml filtrate was added to 10 ml potassium dichromate and absorbance was recorded at 360 nm after development of colour.
- **Niacin**: 5 g of sample was treated with 50 ml of 1 N sulphuric acid for 30 min and 0.5 ml of ammonia solution. It was filtered, to 10 ml of this filtrate 5 ml of potassium cyanide was added and then acidified with 5 ml 0.02 N sulphuric acid. The absorbance of the resulting solution was recorded at 420 nm.
- **Riboflavin**: 5 g sample was extracted with 100 ml ethanol for 1 h. To this 10 ml of this filtered extract, 10 ml 5% potassium permanganate and 10 ml 30% hydrogen peroxide was added and allowed to stand on hot water bath for 30 min. To this 2 ml of 40% sodium sulphate was added. The volume was made up to 50 ml and absorbance was recorded at 510 nm.
- **Ascorbic acid**: Accurately, 1 g of each sample was weighed in a 25 ml conical flask. Then 10 ml of the oxalic acid (0.05 M)-EDTA (0.02 M) solution was added and placed in the sample for 24 h, to provide the required reaction time. After 24 h, the samples were filtered through 0.45 μm filter paper. Then 2.5 ml of each sample was transferred to a separate 25 ml

volumetric brown flask, after which 2.5 ml of the oxalic acid (0.05 M)-EDTA (0.02 M) solution was added. Subsequently, meta phosphoric acid was added separately with acetic acid (0.5 ml), sulphuric acid (5% v/v) solution (1 ml) and ammonium molybdate solution (2 ml) in each volumetric brown flask and the volume was made up to 25 ml with distilled water. The absorbance was measured at 760 nm on a UV/visible spectrophotometer. (Iqbal Hussain. *et al*., **2011**)

$$\text{Concentration} = \frac{\text{Test absorbance}}{\text{Std absorbance}} \times \frac{\text{wt of Test}}{\text{wt of std}} \times \frac{\text{Dilution factor of std}}{\text{Dilution factor of Test}}$$

4.5. Preparation of total ash:

One gram of powdered sample from each Hibiscus species flower were taken in silica crucible and heated over a low Bunsen flame to volatilize as much of the organic material as possible. The crucible was then transferred to a temperature controlled muffle furnace. The temperature was maintained at about 300º C for 5-7 h. It was removed from the muffle furnace and allowed to cool. After cooling, the weight of the ash was noted and the percentage yield was calculated. (Shanthi P. *et al*., **2010**)

$$\% \text{ of Total ash} = \frac{\text{Weight of total ash}}{\text{Weight of powder taken}} \times 100$$

4.6. Qualitative screening of inorganic elements in flower of Hibiscus species:

(Khandelwal K.R. **2008**)

4.6.1. Test for inorganic elements:

To the ash of the powder material, 50% v/v hydrochloric acid or 50% v/v nitric acid was added and kept for 1 h and filtered, to the filtrate the following test were performed.

- **Test for calcium:**

(a)To 10 ml filtrate, one drop dil. ammonium hydroxide and saturated ammonium oxalate solution were added. White precipitate of calcium oxalate forms which was soluble in hydrochloric acid but insoluble in acetic acid.

(b)With the solution of ammonium carbonate gives white precipitate which was insoluble in ammonium chloride solution.

- **Test for iron:**

(a)To 5 ml test solution, few drops of 2% potassium ferrocyanide were added. Dark blue colouration observed.

(b)To 5 ml test solution, few drops 5% ammonium thiocyanate solution was added, turns blood red colour.

- **Test for sulphate:**

(a)To 5 ml filtrate, few drops 5% barium chloride solution was added; white crystalline barium sulphate precipitate appears which was insoluble in hydrochloric acid.

(b)With lead acetate solution gives white precipitate which was soluble in sodium hydroxide.

- **Test for phosphate:** To 5 ml test solution, few drops ammonium molybdate solution was added. It was heated for 10 min and cooled, yellow crystalline precipitate of ammonium phosphormolybdate was observed.
- **Test for magnesium:** Gives white precipitate with ammonium carbonate solution but not with ammonium chloride solution.

- **Test for sodium:**

(a)Flame test: Thick paste from ash was prepared with concentrated hydrochloric acid. The paste on platinum wire loop was introduced in Bunsen flame. Gives golden yellow flame.

(b) To 10 ml of ash solution, 2 ml of potassium pyroanthllollate was added, gives white precipitate.

- **Test for potassium:**

(a)To 2-3 ml of test solution, few drops sodium cobalt nitrite solution was added, yellow precipitate of potassium cobalt nitrite was observed.

(b)Flame test: Gives violet colour to the flame.

- **Test for carbonate:**

(a)With mercuric chloride solution produces a reddish brown precipitate**.**

(b)With solution of magnesium sulphate, white precipitate was formed.

- **Test for nitrate:**

(a)Liberate red fumes when warmed with sulphuric acid and copper.

(b)With solution of ferrous sulphate yield no brown colour but when sulphuric acid added (slowly from side of the test tube) a brown colour was produced at the junction of the two liquids.

4.7. Estimation of inorganic elements in flower of Hibiscus species by AAS method: (Myatt Hla Wai. *et al*., **2005**).

4.7.1 Assay for Calcium:

Preparation of standard solution:

Quantitatively a volume of calcium standard stock solution was diluted with 0.125 N hydrochloric acid to obtain a standard solution having a known concentration of about 100 µg of calcium per ml. It was transferred to separate 100 ml volumetric flasks; 3 ml of the standard solution was pipetted out. To each flask 1 ml of lanthanum chloride solution was added, diluted with water to volume, and mixed to obtain solutions having known concentrations of about 3 µg of calcium per ml.

Preparation of sample solution:

1 g of sample was transferred in a crucible in a muffle furnace maintained at about 550° C for 6 to 12 h, and cool. About 60 ml of hydrochloric acid was added, and gently boiled on a hot plate or steam bath for 30 min, intermittently rinsing the inner surface of the crucible with 6 N hydrochloric acid. It was cooled, and quantitatively the contents of the crucible were transferred to a 100 ml volumetric flask. The crucible was rinsed with small portions of 6 N hydrochloric acid, and added the rinsings to the flask. Then it was diluted with water to volume, mixed, and filtered, discarding the first 5 ml of the filtrate. This solution was diluted quantitatively and stepwise if necessary, with 0.125 N hydrochloric acid to obtain a solution having a concentration of about 2 µg of calcium per ml, adding 1 ml of lanthanum chloride solution per 100 ml of the final volume.

Procedure:

concurrently the absorbance of the standard preparations and the assay preparation was determined at the calcium emission line at 422.7 nm with an atomic

absorption spectrophotometer equipped with calcium hollow-cathode lamp and a nitrous oxide-acetylene flame, using 0.125 N hydrochloric acid containing 0.1% lanthanum chloride solution as the blank in µg per ml, of calcium in the assay preparation. The weight, in mg, of calcium (Ca) was calculated in the portion of sample.

4.7.2 Assay for iron:

Preparation of standard solution:

To separate 100 ml volumetric flasks, 2.0, 4.0, 5.0, 6.0, and 8.0 ml of vandadium standard stock solution was transferred. The contents of each flask was diluted with water to volume, and mixed to obtain solutions having known concentrations of about 2.0, 4.0, 5.0, 6.0, and 8.0 µg of iron per ml.

Preparation of sample solution:

Assay preparation to contain 5 µg of vandadium per ml and to omit the use of the lanthanum chloride solution.

Procedure:

The absorbance of the standard preparations and the assay preparation was determined at the iron emission line at 248.3 nm with an atomic absorption spectrophotometer equipped with an iron hollow-cathode lamp and an air-acetylene flame, using 0.125 N hydrochloric acid as the blank. The absorbance of the standard preparations versus concentration, in µg per ml of iron was plotted, and the straight line best fitting the five plotted points was drawn. From the graph so obtained, the concentration, C, in µg per ml, of iron in the assay preparation was determined and the quantity, in mg, of vandadium in the portion of ash taken was calculated by the

formula: 0.001CD, in which D is the dilution factor used to prepare the assay preparation.

4.7.3 Assay for phosphorus:

Preparation of standard solution:

An accurately measured volume of phosphorus standard was dissolved to stock solution in water, and quantitatively diluted with water to obtain a solution having a known concentration of about 20 µg of phosphorus per ml.

Preparation of sample solution:

Accurately weighed portion of the ash, equivalent to about 100 mg of phosphorus, to a suitable flask, 25 ml nitric acid was added, and digested on a hot plate for about 30 min. 15 ml of hydrochloric acid was added, and the digestion was continued until the cessation of brown fumes. It was cooled, and quantitatively transferred the contents of the flask to a 500 ml volumetric flask with the aid of small portions of water. Then it was diluted with water to volume, and mixed. 10 ml of this solution was transferred to a 100 ml volumetric flask, diluted with water to volume, and mixed.

Procedure:

To three separate 25 ml volumetric flasks, 5 ml each of the standard preparation was transferred, the assay preparation, and water to provide the blank. To each of the three flasks, 1 ml each of ammonium molybdate solution, hydroquinone solution, and Sodium bisulfate solution were added, and swirled to mix. The contents of each flask was diluted with water to volume, mixed, and allowed the flasks to stand for 30 min. Concomitantly the absorbance of the solutions from the assay preparation and the Standard preparation in 1 cm cells at the wavelength of maximum absorbance

at about 650 nm, against the blank was determined. The quantity, in mg, of phosphorus (P) in the portion of ash taken was calculated by the formula: 5C (AU/AS), in which C is the concentration, in µg per ml of phosphorus in the standard preparation; and A U and A S are the absorbance of the solutions obtained from the assay preparation and the Standard preparation, respectively.

4.8. Proximate analysis in flower of Hibiscus species: (Indian Pharmacopeia., **1985)**

4.8.1 Sulphated ash:

The silica crucible was heated to redness for 10 min, cooled in a desicator and weighed. 1 g of substance accurately weighed into the crucible, ignited gently first until the substances are thoroughly charred. Cooled, moisten the residue with 1 ml of sulphuric acid, heated gently until white fumes are no longer evolved and ignited at 800°±25° C until all black particles have disappeared, conduct the ignition in a place protected from air currents. The crucible was cooled and few drops of sulphuric acid was added and heated. Ignited as before, allowed to cool and weighed.

4.8.2. Acid insoluble ash:

Total ash obtained was heated with 25 ml of 2 N hydrochloric acid for 5 min, collected the insoluble matter on an ash less filter paper, washed with hot water, ignited and weighed. The percentage of acid insoluble ash was calculated with reference to the air dried sample.

4.8.3. Water soluble ash:

The ash was boiled for 5 min with 25 ml of water, collected the insoluble matter in a ash less filter paper, washed with hot water and ignited for 15 min at temperature not exceeding 450° C. subtracted the weight of the insoluble matter from

the weight of the ash, the difference in the weight represents the water soluble ash. The percentage of water soluble ash was calculated with reference to the air dried sample.

4.9. TLC study in methanolic flower extracts of Hibiscus species: (Chakraborthy Guno Sindhu. **2010**)

Thin layer chromatography (TLC) was employed in this study to analyze the nature of compounds present in the crude methanolic flower extracts of Hibiscus species. Stationary phase silica gel G (scored 10×20 cm) plates, were used. Each flower extracts of Hibiscus species was applied as three separate spots on the TLC plates about 1.3 cm above from the edge (spotting line), using 20 µl capillary tubes (microcaps disposable pipettes, Drummond Scientific Company) and developed in the following solvent system :

(i) Butanol:ethylacetate:water (10:10:4)

(ii) Butanol:aceticacid:water (12:3:5)

(iii) Butanol:aceticacid:water (14:1:5)

(iv) Ethylacetate: methanol: water (5:1.1:1) and detected by following methods:

(a) Chamber saturated with iodine vapours.

(b) Sprayed with 0.2% Ninhydrin in acetone.

(c) Spraying the plates with Vanillin-sulphuric acid reagent and detected in UV light.

The R_f value of individual spot was calculated by using the following formula:

$$R_f = \frac{\text{Distance travelled by solute}}{\text{Distance travelled by solvent front}}$$

4.10. UV and FT-IR fingerprinting in methanolic flower extracts of Hibiscus species: (Padma S. *et al.,* **2008**)

Each flower extracts of Hibiscus species were dissolved in methanol in microgram quantities. Their UV- Visible spectra were scanned at the range of 200-800 nm by using Shimadzu 1601 UV-spectrophotometer. In each case the base line was cleared against the solvent in which the particular solution of extract was prepared. The scanned spectra were recorded and peaks of maximum absorption were noted.

Each flower extracts of Hibiscus species (10 mg) was mixed with 100 mg of dried potassium bromide (KBr) and compressed to prepare as a salt disc. The disc was then read spectrophotometrically in the range of 400 cm^{-1} to 4000 cm^{-1} by using Shimadzu FTIR-8101 spectrophotometer (V_{max} in cm^{-1}). The frequencies of different components present in each extract were analyzed.

4.11. HPTLC Finger printing in methanolic flower extracts of Hibiscus species: (Gupta V. *et al.,* **2009)**

The HPTLC finger printing of flower extract of each species of Hibiscus was determined in the following condition:

Instruments condition:

Source	: AHRF/HPTLC/C09/DE/REPORTS/280
Mobile Phase	: n-butanol: Ethyl acetate: water (10:10:4)
Lamp	: Deuterium Lamp (254nm)
Sample volume loaded	: 20µl
Applicator	: CAMAG Linomat IV
Scanner	: CAMAG TLC SCANNER II

4.12. GC-MS analysis in methanolic flower extracts of Hibiscus species: (Raja Rajeswari N. *et* al., **2011**)

The chemical characterization of three species of the methanolic flower extracts of Hibiscus was carried out by using Clarus 50 Perkin Elmer Gas Chromatography in the following condition:

Condition:

Column Oven Temperature : 70˚ C

Injector Temperature : 240˚ C

Injection Mode : Split

Split Ratio : 10

Flow Control Mode : Linear Velocity

Column Flow : 1.51 ml/min

Carrier Gas : Helium 99.9995% purity

Injection volume : 1 microlitre

Column

Length : 30.0 m

Diameter : 0.25 mm

Film thickness : 0.25 µm

Ms Condition:

Ion source temperature : 200° C

Interface temp : 240° C

Scan range : 40 – 1000 m/z

Ionization : EI (-70ev)

Scan speed : 2000

Identification:

The identity of the components in the extracts were assigned by the comparison of their retention indices and mass spectra fragmentation patterns with

those stored on the computer library and also with published literatures. NIST08.LIB, WILEY8.LIB, and FAME.LIB library sources were used for matching the identified components from the plant material.

4.13. Screening of antimicrobial activity in methanolic flower extracts of Hibiscus species: (Sangeetha Arullapan . *et al*., **2009)**

The prepared extracts from flower of each species of Hibiscus were tested for antimicrobial activity by disc diffusion method. They were dissolved in DMSO and sterilized by filtering through 0.45 µm millipore filter. The final inoculums of 100 µl suspension containing 10^8 CFU/ ml of each bacterium and fungus used. Nutrient agar (antibacterial activity) and sabouraud's dextrose agar medium (antifungal activity) was prepared and sterilized by an autoclave (121º C and 15 Ibs for 20 min) and transferred to previously sterilized petridishes (9 cm in diameter). After solidification, petriplates were inoculated with bacterial organisms in sterile nutrient agar medium at 45^o C, and fungal organism in sterile sabouraud's dextrose agar medium at 45^o C in aseptic condition. Sterile Whatmann filter paper discs (previously sterilized in U.V. lamp) was impregnated with prepared extracts at a concentration of 25, 100 µg /disc were placed in the organism impregnated petri plates under sterile condition. The plates were left for 30 min to allow the diffusion of extracts at room temperature. Antibiotic discs of ciprofloxacin (100 µg /disc) and ketaconazole (100 µg /disc) were used as positive control, while DMSO used as negative control. Then the plates were incubated for 24 h at 37 ± 1^o C for antibacterial activity and 48 h at 37±1^o C for antifungal activity. The zone of inhibition was calculated by measuring the minimum dimension of the zone of no microbial growth around the disc.

RESULTS
&
DISCUSSION

5. RESULTS AND DISCUSSION

5.1. Physical properties of flower extracts of Hibiscus species:

The dried flower powder of each Hibiscus species were subjected to petroleum ether, ethyl acetate and methanol cold extraction and the percentage yield of petroleum ether extract of *Hibiscus rosa sinensis*, *Hibiscus syriacus* and *Hibiscus arnottianus* were 18.2%, 21.5% and 21.3% whereas the ethyl acetate extract of *Hibiscus rosa sinensis*, *Hibiscus syriacus* and *Hibiscus arnottianus* were 19.5%, 22.8% and 24.8% and the methanolic extract of *Hibiscus rosa sinensis*, *Hibiscus syriacus* and *Hibiscus arnottianus* were 30.76%, 36.2% and 32.65, the organoleptic evaluation of the extracts such as colour, odour and consistency were also observed and are reported in Table.5.1, 5.2 and 5.3.

Table.5.1. Physical properties of the flower extract of *Hibiscus rosa sinensis*

S.No	Physical characteristics	Extracts		
		Petroleum ether	**Ethyl acetate**	**Methanol**
1.	Colour	Reddish brown	Pink red	Brownish black
2.	Odour	Characteristic	Characteristic	Characteristic
3.	Consistency	Viscous semisolid	Viscous semisolid	Viscous semisolid
4.	Percentage yield	18.2 %	19.5 %	30.76%

Table.5.2. Physical properties of the flower extract of *Hibiscus syriacus*

S.No	Physical characteristics	Extracts		
		Petroleum ether	**Ethyl acetate**	**Methanol**
1.	Colour	Brown	Pinkish red	Pinkish red
2.	Odour	Characteristic	Characteristic	Characteristic
3.	Consistency	Viscous semisolid	Viscous semisolid	Viscous semisolid
4.	Percentage yield	21.5%	22.8%	36.2%

Table.5.3. Physical properties of the flower extract of *Hibiscus arnottianus*

S.No	Physical characteristics	Extracts		
		Petroleum ether	**Ethyl acetate**	**Methanol**
1.	Colour	Brownish black	Brown	Brownish black
2.	Odour	Characteristic	Characteristic	Characteristic
3.	Consistency	Viscous semisolid	Viscous semisolid	Viscous semisolid
4.	Percentage yield	21.3%	24.8%	32.6%

5.2. Phytochemicals and vitamin analysis in methanolic flower extracts of Hibiscus species:

The preliminary phytochemical investigation of the methanolic extracts of Hibiscus species showed the presence of carbohydrates, proteins, amino acids, glycosides, flavonoids, tannins, steroids and phenolic compounds. The flower extracts also showed the presence of vitamins such as thiamine, niacin, riboflavin and ascorbic acid and these phytochemicals and vitamins were quantitatively evaluated and given in Table.5.4 & 5.5.

Table.5.4. Qualitative screening of phytochemicals and vitamins in methanolic flower extracts of Hibiscus species

S.No	Phytochemicals/ vitamins	*Hibiscus rosa sinensis*	*Hibiscus syriacus*	*Hibiscus arnottianus*
1.	Carbohydrates	+	+	+
2.	Proteins and amino acids	+	+	+
3.	Alkaloids	–	–	–
4.	Glycosides	+	+	+
5.	Saponin glycoside	–	–	–
6.	Anthraquinone glycoside	–	–	–
7.	Cardiac glycoside	–	–	–
8.	Flavonoids	+	+	+
9.	Phenolic	+	+	+
10.	Tannins	+	+	+
11.	Steroids	+	+	+
12.	Fats & fixed oils	+	+	+
13.	Thiamine	+	+	+
14.	Niacin	+	+	+
15.	Riboflavin	+	+	+
16.	Ascorbic acid	+	+	+

(+) = Indicates presence (-) = Indicates absence

Table.5.5. Quantitative estimation of phytochemicals and vitamins in methanolic flower extracts of Hibiscus species.

S.No	Phytochemicals and vitamins	*Hibiscus rosa sinensis*	*Hibiscus syriacus*	*Hibiscus arnottianus*
1.	Flavonoids	0.171 mg/g	0.278 mg/g	0.212 mg/g
2.	Total phenolic	0.092 mg/g	0.1 mg/g	0.098 mg/g
3.	Tannins	0.073 mg/g	0.089 mg/g	0.083 mg/g
4.	Carbohydrate	0.427 mg/g	0.686 mg/g	0.612 mg/g
5.	Protein	6.827 mg/g	7.234 mg/g	6.984 mg/g
3	Thiamine	0.072 mg/g	0.069 mg/g	0.056 mg/g
4	Niacin	0.075 mg/g	0.060 mg/g	0.053mg/g
5	Ascorbic acid	0.0339 mg/g	0.0193 mg/g	0.0158 mg/g
6.	Riboflavin	0.087 mg/g	0.082 mg/g	0.074 mg/g

5.3. Proximate and elemental analysis in flower of Hibiscus species:

The results of proximate analytical parameter such as total ash, acid insoluble, water soluble and sulphated ash were reported in Table.5.6. The percentage yield of total ash and sulphated ash for *Hibiscus rosa sinensis*, *Hibiscus syriacus* and *Hibiscus arnottianus* were found to be 17.8%, 17.9%, 18.2% and 4.7%, 4.21%, 3.6% respectively. Similarly, the percentage yield of acid insoluble and water soluble ash for *Hibiscus rosa sinensis*, *Hibiscus syriacus* and *Hibiscus arnottianus* were found to be 6.6%, 7.34%, 7.40% and 29.41%, 21.14%, 20.5% respectively. From the chemical test on ash, all the three species showed the presence of calcium, iron and phosphorus and these elements were quantitatively estimated by Atomic Absorption Spectroscopy and listed in Table.5.7 & 5.8.

Table.5.6. Proximate analysis in flower of Hibiscus species

S.No	Parameter	*Hibiscus rosa sinensis*	*Hibiscus syriacus*	*Hibiscus arnottianus*
1.	Total ash & colour	17.8%	17.9%	18.2%
		Pale brown	Pale brown	Pale brown
2.	Acid insoluble ash	6.6%	7.34%	7.40%
3.	Water soluble ash	29.41%	21.14%	20.5%
6.	Sulphated ash	4.7%	4.21%	3.6%

Table.5.7. Qualitative screening of inorganic elements in flower of Hibiscus species

S.No	Elements	*Hibiscus rosa sinensis*	*Hibiscus syriacus*	*Hibiscus arnottianus*
1.	Calcium	+	+	+
2.	Magnesium	–	–	–
3.	Sodium	–	+	+
4.	Potassium	–	+	+
5.	Iron	+	+	+
6.	Sulphate	–	–	–
7.	Phosphorus	+	+	+
8.	Chloride	–	–	–
9.	Carbonate	–	–	–
10.	Nitrate	–	–	–

(+) = Indicates presence (-) = Indicates absence

Table.5.8. Elemental analysis in flower of Hibiscus species by AAS

S.No	Hibiscus species	Calcium	Iron	Phosphorus
1.	*Hibiscus rosa sinensis*	0.0127%	0.771%	0.4113%
2.	*Hibiscus syriacus*	0.0136%	0.7216%	0.4342%
3.	*Hibiscus arnottianus*	0.0142%	1.058%	0.582%

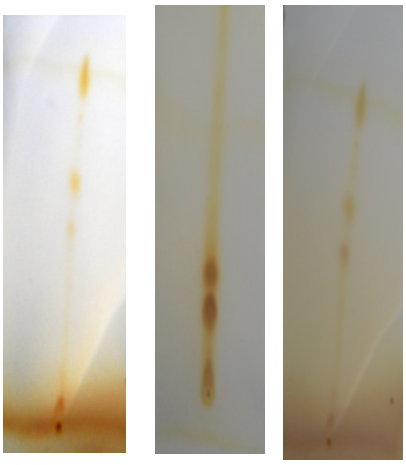
5.4. TLC chromatogram in methanolic flower extracts of Hibiscus species:

The TLC chromatographic profile of the methanolic flower extracts of Hibiscus species was developed with various developing and visualizing agents and the results were reported in Table.5.9.

- The extracts of Hibiscus species was developed in n-butanol: ethyl acetate: water (10:10:4) using iodine vapour as visualizing agent. *Hibiscus rosa sinensis* showed 4 spots with the R_f values 0.79, 0.86, 0.27 and 0.04, *Hibiscus syriacus* showed 5 spots with the R_f values 0.74, 0.70, 0.39, 0.18 and 0.48 and *Hibiscus arnottianus* showed 3 spots R_f values 0.24, 0.16 and 0.05.
- The presence of amino acids was confirmed by the development of purple colour spots on spraying with ninhydrin solution using n-butanol: acetic acid: water: (12:3:5). *Hibiscus rosa sinensis* showed one spot with R_f value 0.82, *Hibiscus syriacus* showed at 0.96 and *Hibiscus arnottianus* at 0.70.
- The flower extracts of *Hibiscus rosa sinensis* gave one spot with R_f value 0.89 in butanol: acetic acid: water: (14:1:5) under UV light, *Hibiscus syriacus* showed at 0.88 and *Hibiscus arnottianus* at 0.96 which confirms for flavonoids.
    - In case of steroids *Hibiscus rosa sinensis* showed 3 spots with R_f values 0.24, 0.16 and 0.05 using vanillin-sulphuric acid as spraying agents whereas *Hibiscus syriacus* showed two spots with R_f value 0.99 and 0.08 and *Hibiscus arnottianus* showed 2 spots with Rf values 0.69 and 0.13 was given in fig. 5.1.

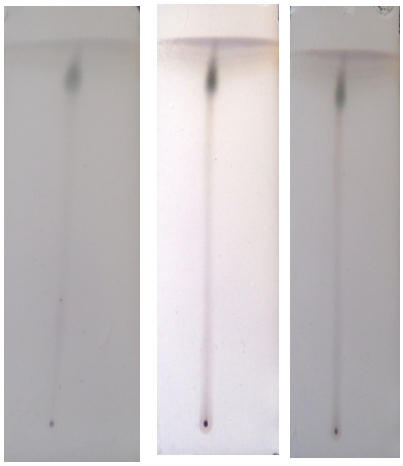
Table.5.9. TLC chromatogram in methanolic flower extracts of Hibiscus species

Solvent system	Number of spots and R_f values			Detecting agent	Colour of spot
	Hibiscus rosa sinensis	***Hibiscus syriacus***	***Hibiscus arnottianus***		
Butanol:Ethylacetate:Water (10:10:4)	4 (0.79,0.86,0.27,0.04)	5 (0.74,0.70, 0.39,0.18, 0.48)	3 (0.24,0.16,0.05)	Iodine vapour	Brown
Butanol:Aceticacid:Water (12:3:5)	1(0.82)	1(0.96)	1(0.70)	Ninhydrin	Violet colour
Butanol:Aceticacid:Water (14:1:5)	1(0.89)	1(0.88)	1(0.96)	UV at 254	Dark purple
Ethylacetate:Methanol:Water (5:1.1:1)	3(0.24,0.16,0.05)	2(0.99,0.08)	2(0.69,0.13)	Vanillin-sulphuric acid	Yellow colour



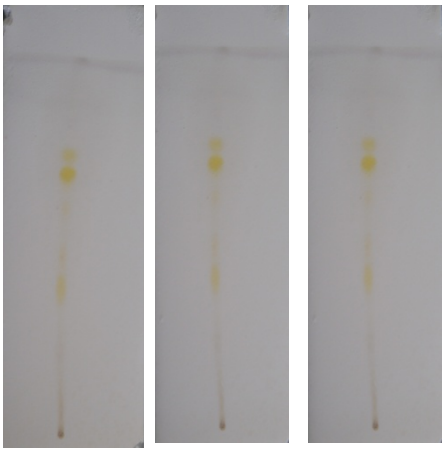
A B C

(a) Iodine vapour



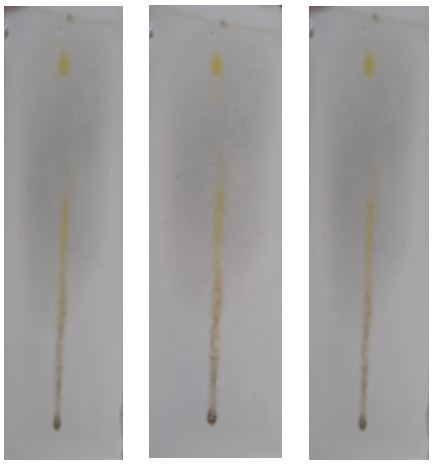
A B C

(b) 0.2% Ninhydrin



A B C

(c) UV light at 254 nm



A B C

(d) Vanillin-sulphuric acid

A – *Hibiscus rosa sinensis*

B – *Hibiscus syriacus*

C – *Hibiscus arnottianus*

Fig.5.1 TLC chromatogram in methanolic flower extracts of Hibiscus species

5.5. UV Finger printing in methanolic flower extracts of Hibiscus species:

Table.5.10. UV absorbance in methanolic flower extracts of Hibiscus species

S.No	Sample	λ_{max}	Absorbance
1.	*Hibiscus rosa sinensis*	272.5 nm	0.2046
2.	*Hibiscus syriacus*	257.0 nm	0.0624
		358.5 nm	0.0459
3.	*Hibiscus arnottianus*	256.5 nm	0.1078
		351.0 nm	0.0696

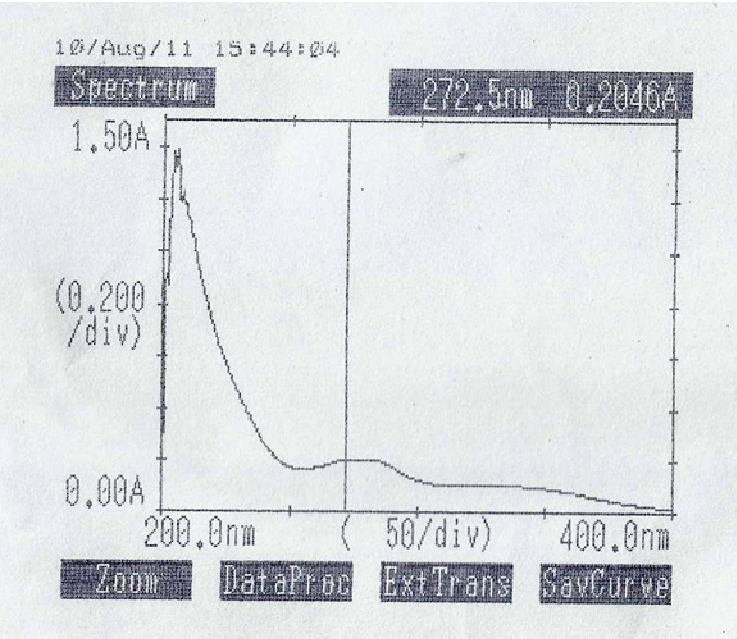


Fig.5.2 UV spectrum of methanolic flower extract of *Hibiscus rosa sinensis*

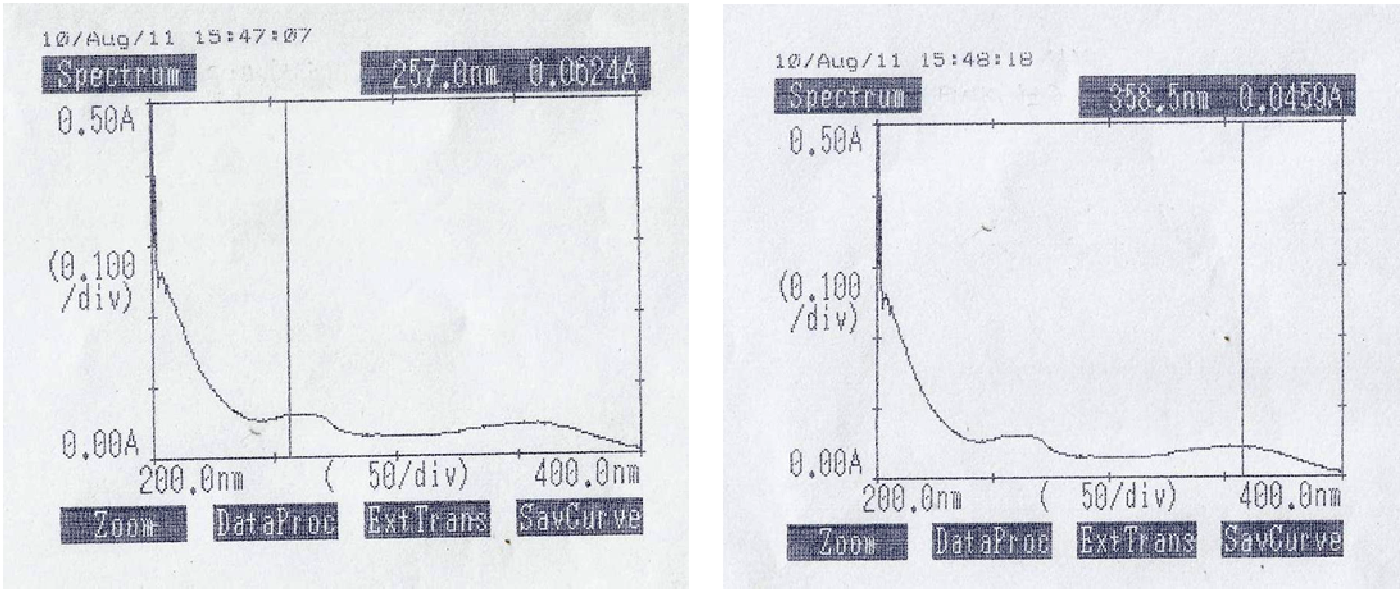


Fig.5.3 UV spectrum of methanolic flower extract of *Hibiscus syriacus*

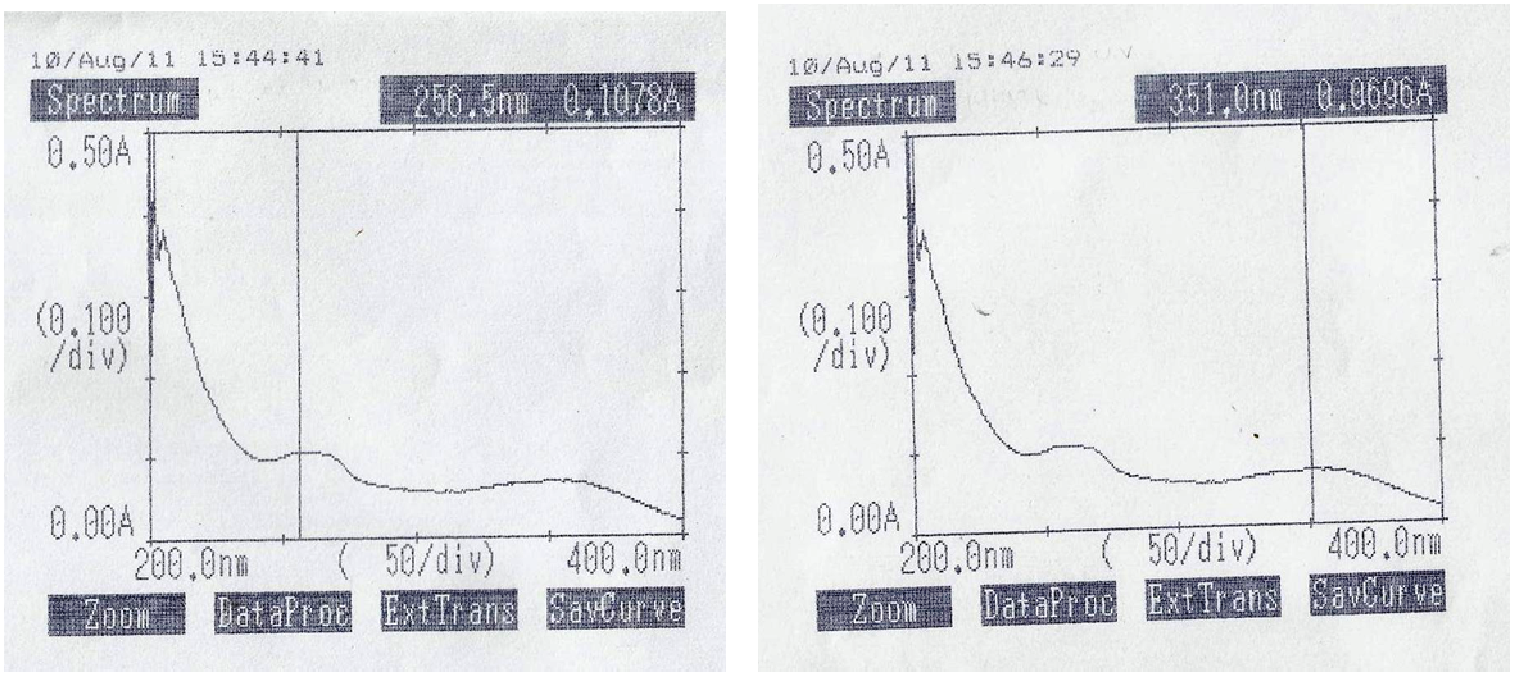


Fig.5.4 UV spectrum of methanolic flower extract of *Hibiscus arnottianus*

5.6. FT-IR fingerprinting in methanolic flower extracts of Hibiscus species:

Table.5.11. FT-IR interpretation in methanolic flower extract of *Hibiscus rosa sinensis*

S.No	Wave number cm^{-1}	Assignment
1.	3396.52	Phenolic O-H stretching
2.	2925.08	C-H stretching for ketones
3.	2852.97	OH stretching
4.	1734.64	C=O stretching
5.	1629.00	C-C stretching for alkenes
6.	1452.49	N-H in plane bending
7.	1401.08	N=O stretching
8.	1340.18	C-N stretching for primary amine
9.	1107.35	N-H stretching
10.	1054.71	S=O stretching for sulphoxide
11.	992.30	C-H out of plane bending for aldehydes
12.	932.08	N-N stretching
13.	890.94	C-H bending for alkanes
14.	899.16	N-H bending for aliphatic primary amine
15.	825.20	S-O stretching
16.	787.03	C-S stretching
17.	718.37	C-Cl stretching
18.	663.59	C-Br stretching

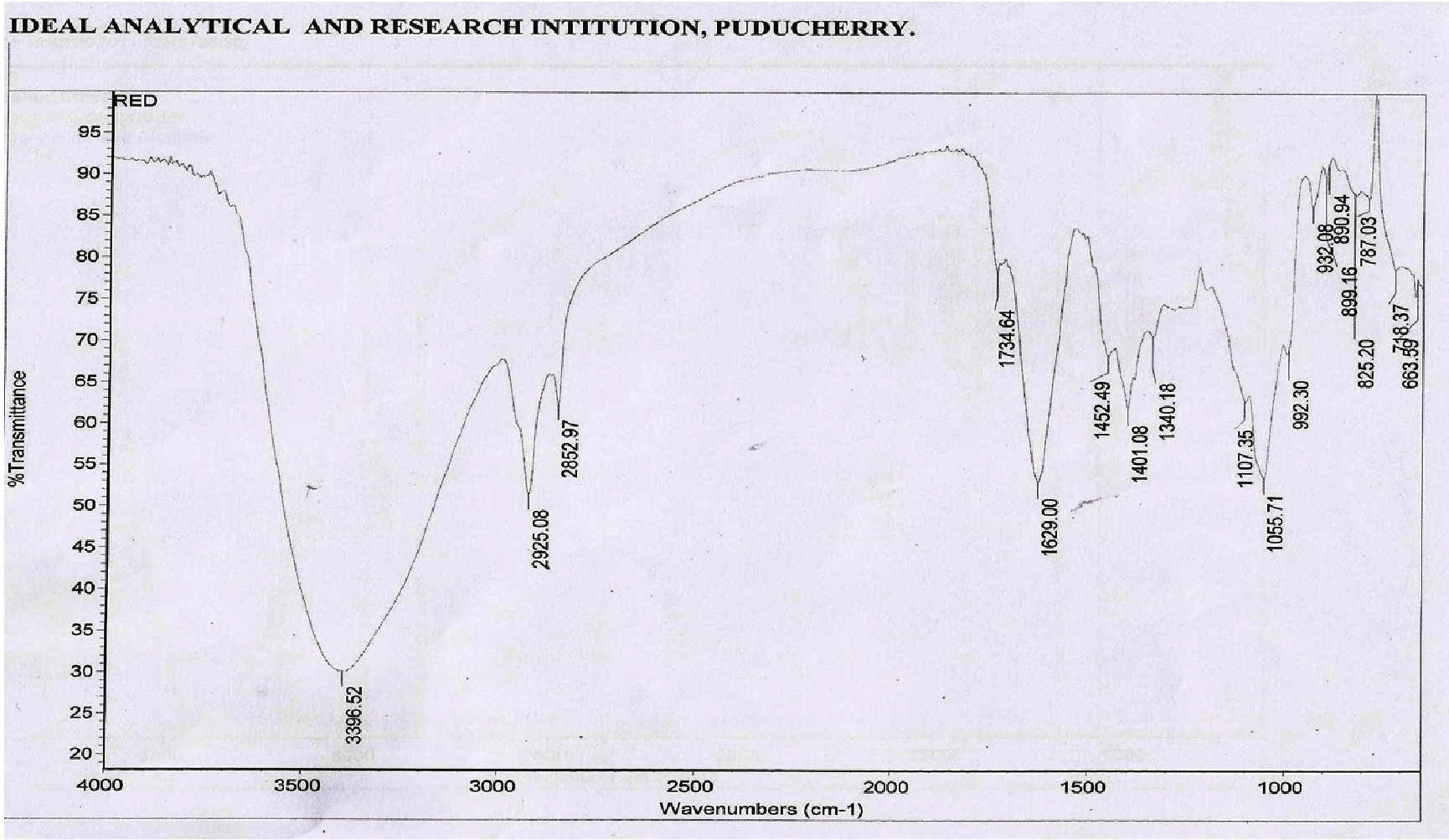


Fig.5.5 FT-IR spectrum in methanolic flower extract of *Hibiscus rosa sinensis*

Table.5.12. FT-IR interpretation in methanolic flower extract of *Hibiscus syriacus*

S.No	Wave number cm^{-1}	Assignment
1.	3386.80	Phenolic O-H stretching
2.	2931.31	C-H stretching for ketones
3.	1628.82	C-C stectching for alkenes
4.	1490.84	N-H symmetric bending
5.	1452.49	N-H in plane bending
6.	1400.51	N=O stretching
7.	1337.44	C-N stretching for primary amine
8.	1233.35	C-CO-C stretching for alkyl ketone
9.	1203.22	C-N stretching for alkyl amine
10.	1056.84	S=O stretching for sulphoxide
11.	992.30	N-N stretching
12.	932.67	N-O stretching for oximes
13.	899.17	C-H bending for alkanes
14.	816.98-671.80	N-H bending for aliphatic primary amine
15.	782.58	C-Cl stretching
16.	671.80	C-Br stretching

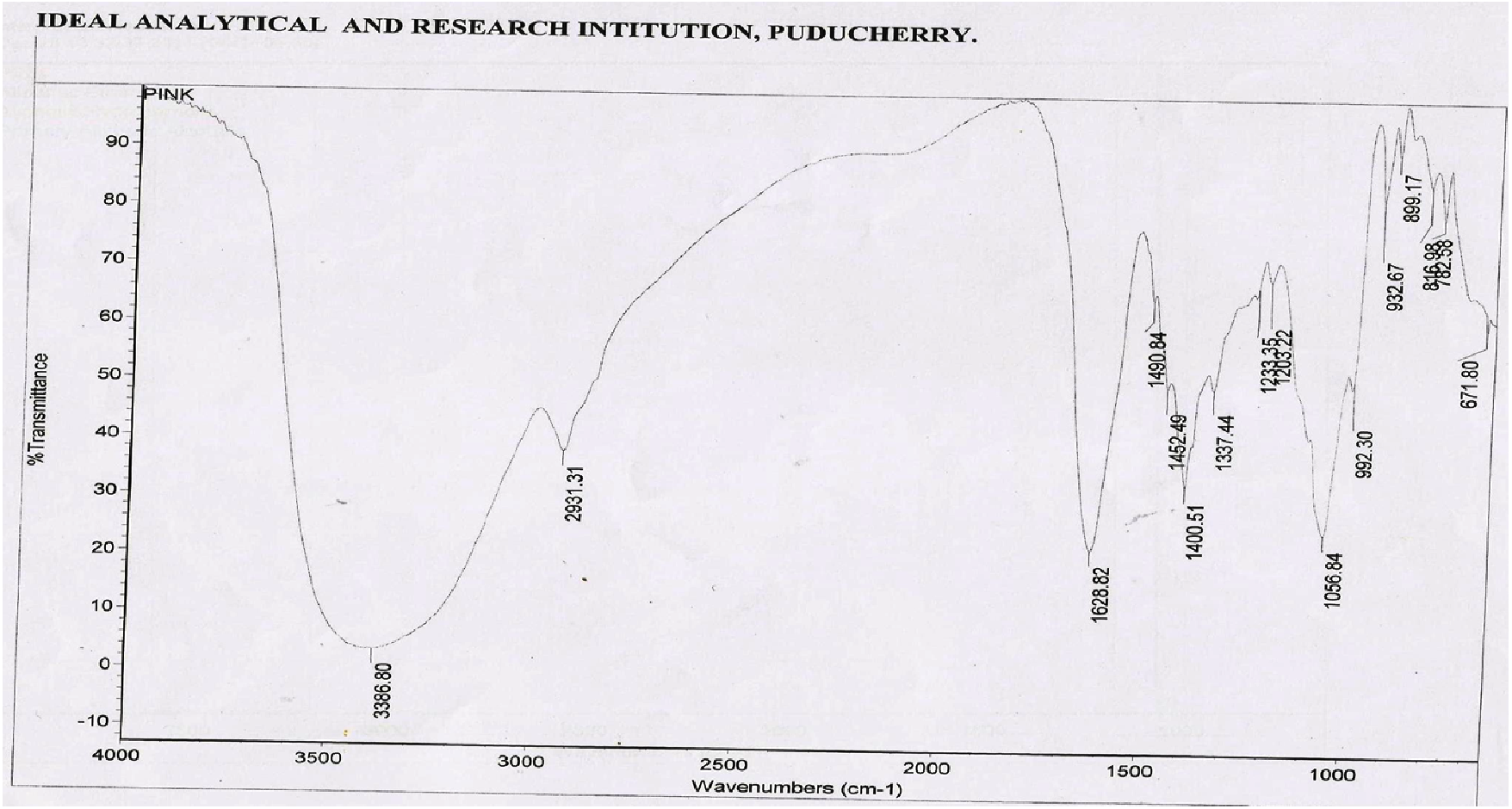


Fig.5.6 FT-IR spectrum in methanolic flower extract of *Hibiscus syriacus*

Table.5.13. FT-IR interpretation in methanolic flower extract of *Hibiscus arnottianus*

S.No	Wave number cm^{-1}	Assignment
1.	3341.32	Phenolic O-H stretching
2.	2932.64	C-H stretching for ketones
3.	1628.9	C-C stectching for alkenes
4.	1442.27	N-H in plane bending
5.	1401.21	N=O stretching
6.	1337.44	C-N stretching for primary amine
7.	1266.22	Asymmetric C-O-C stretching
8.	1236.09	C-CO-C stretching for alkyl ketone
9.	1205.96	C-N stretching of alkyl amine
10.	1057.11	S=O stretching for sulphoxide
11.	992.30	N-N stretching
12.	930.23	N-O stretching for oximes
13.	899.16	C-H bending for alkanes
14.	866.29	C-H out of plane bending for aldehydes
15.	778.11	N-H bending for aliphatic primary amine

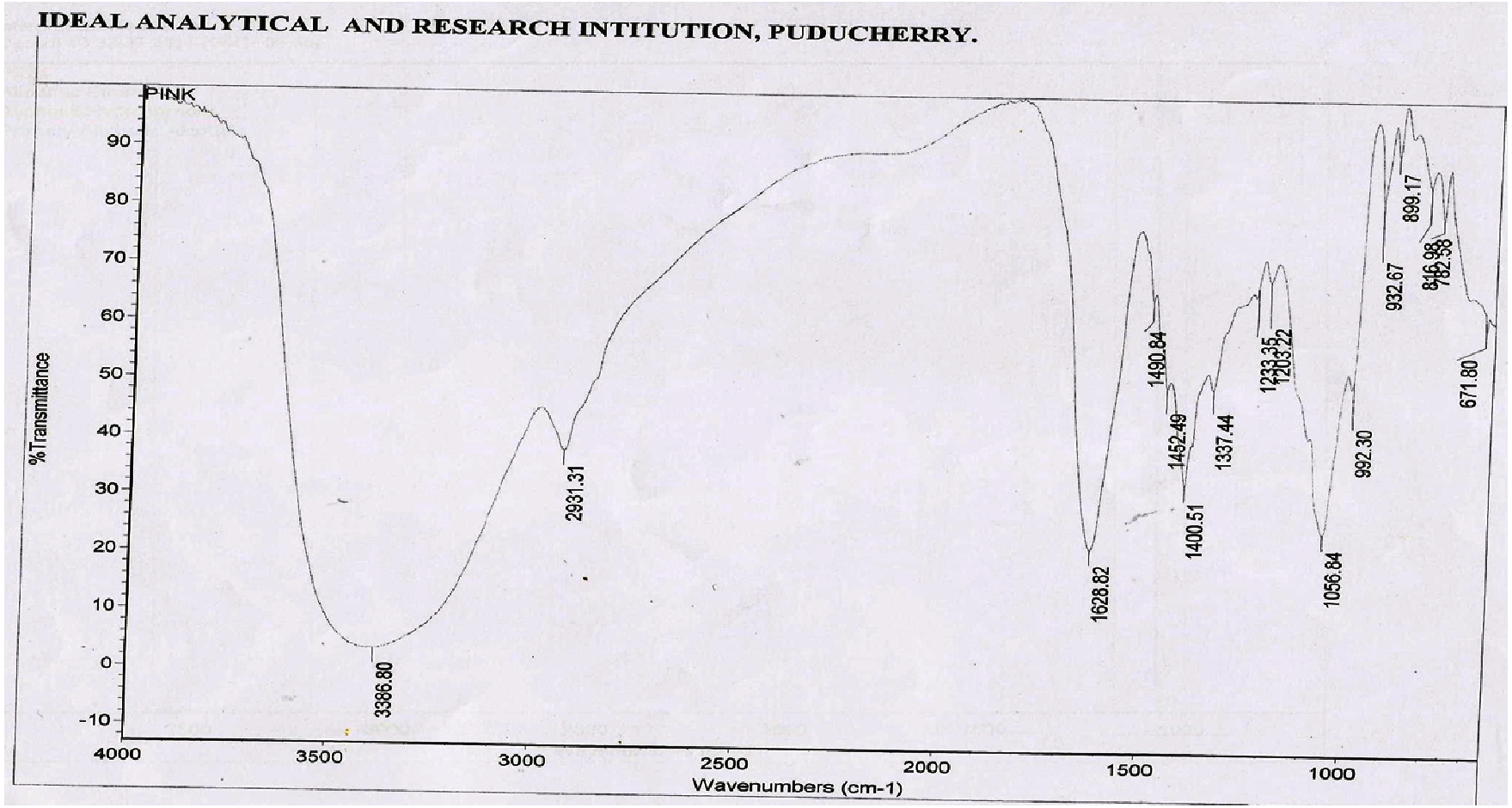
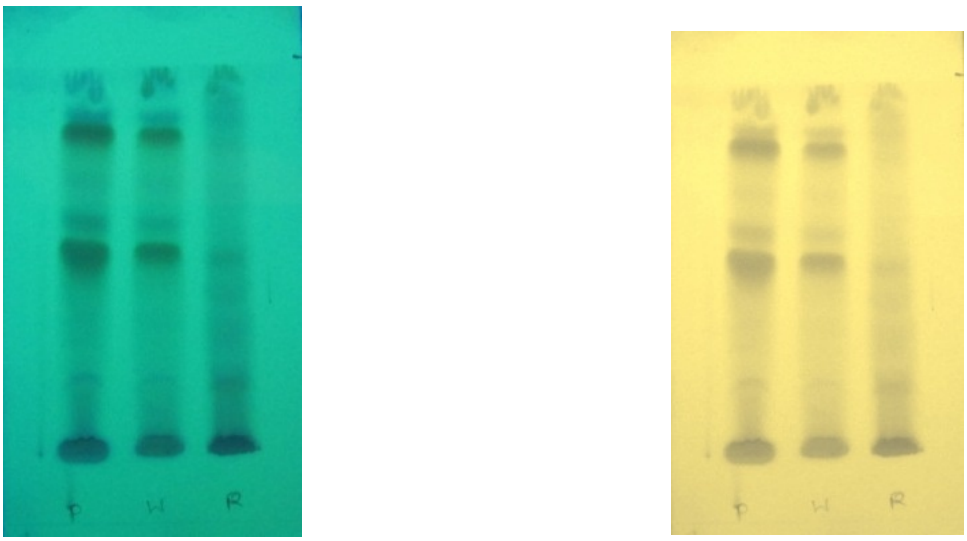


Fig.5.7 FT-IR spectrum in methanolic flower extract of *Hibiscus arnottianus*

5.7. HPTLC fingerprinting in methanolic flower extracts of Hibiscus species:

The HPTLC spectrum of flower extract of *Hibiscus rosa sinensis*, *Hibiscus syriacus* and *Hibiscus arnottianus* were shown in Fig. 5.9, 5.10 and 5.11. In all the Hibiscus species, R_f value of each compound which were separated on plate (Fig. 5.8) and data of peak area and peak height of each band was recorded. In the spectrum of *Hibiscus rosa sinensis* at 254 nm, resolved into 14 peaks (R_f 0.04, 0.11, 0.20, 0.31, 0.33, 0.42, 0.51, 0.56, 0.68, 0.80, 0.88, 0.91, 0.94 & 0.99) with a major compound (26.28%) at R_f 0.04, while the percentage of other peaks was ranging from 0.91 to 13.12%. The scanning of extract from *Hibiscus syriacus* at the same wavelength resolved into 11 peaks (R_f 0.05, 0.22, 0.24, 0.31, 0.37, 0.54, 0.62, 0.73, 0.85, 0.88 & 0.97) with a major compound (22.78%) at R_f 0.05, while the percentage of other peaks was ranging from 3.44 to 16.54%. The extract from *Hibiscus arnottianus* on scanning showed the presence of 13 peaks (R_f 0.04, 0.13, 0.21, 0.23, 0.30, 0.35, 0.53, 0.61, 0.71, 0.83, 0.88, 0.95 & 0.98), the peak at R_f 0.04 was the major one with 19.45%.



A. Under UV 254nm **B. Under Visible (after derivatization)**

Fig.5.8 TLC chromatogram in methanolic flower extracts of Hibiscus species

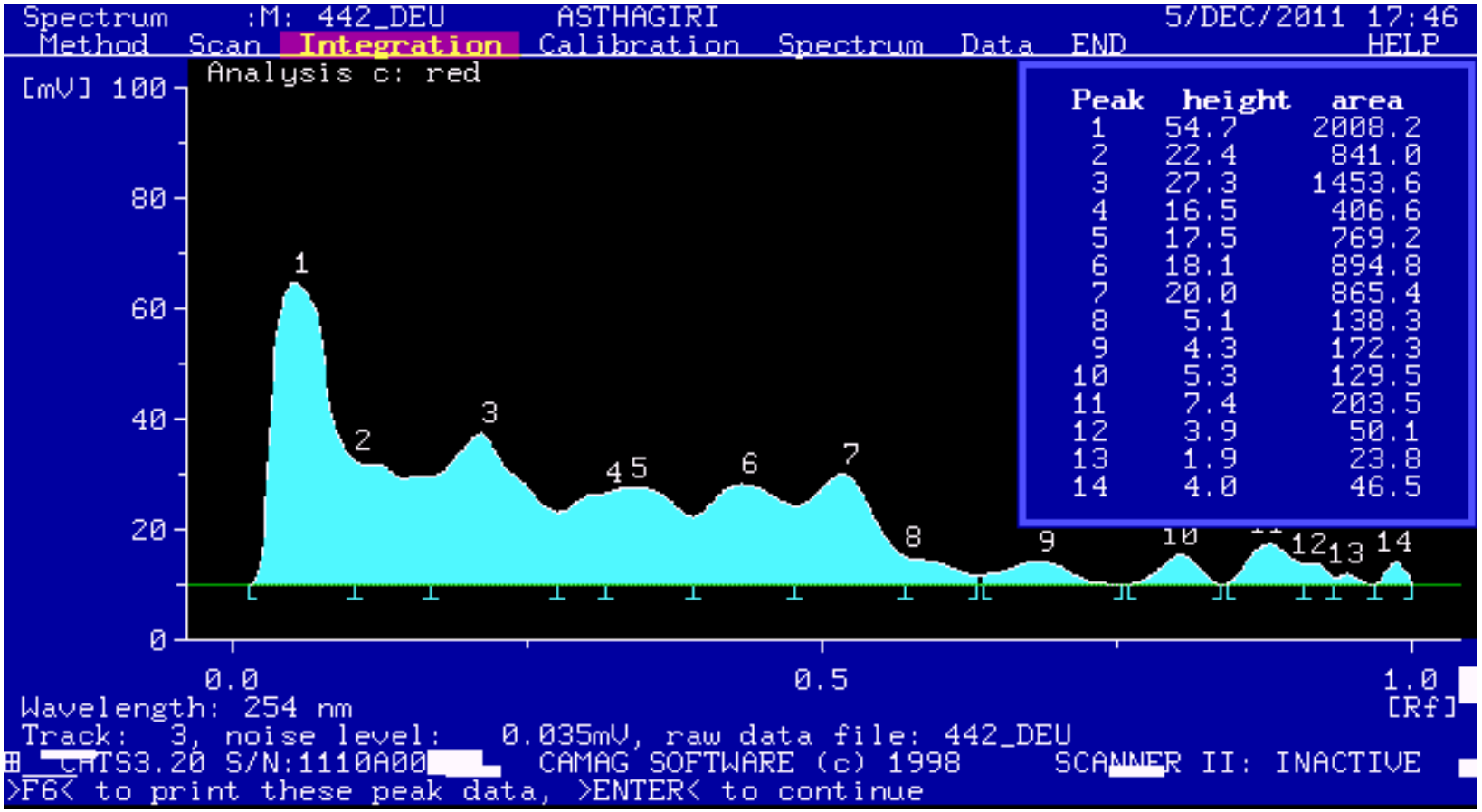


Fig.5.9 HPTLC spectrum in methanolic flower extract of *Hibiscus rosa sinensis*

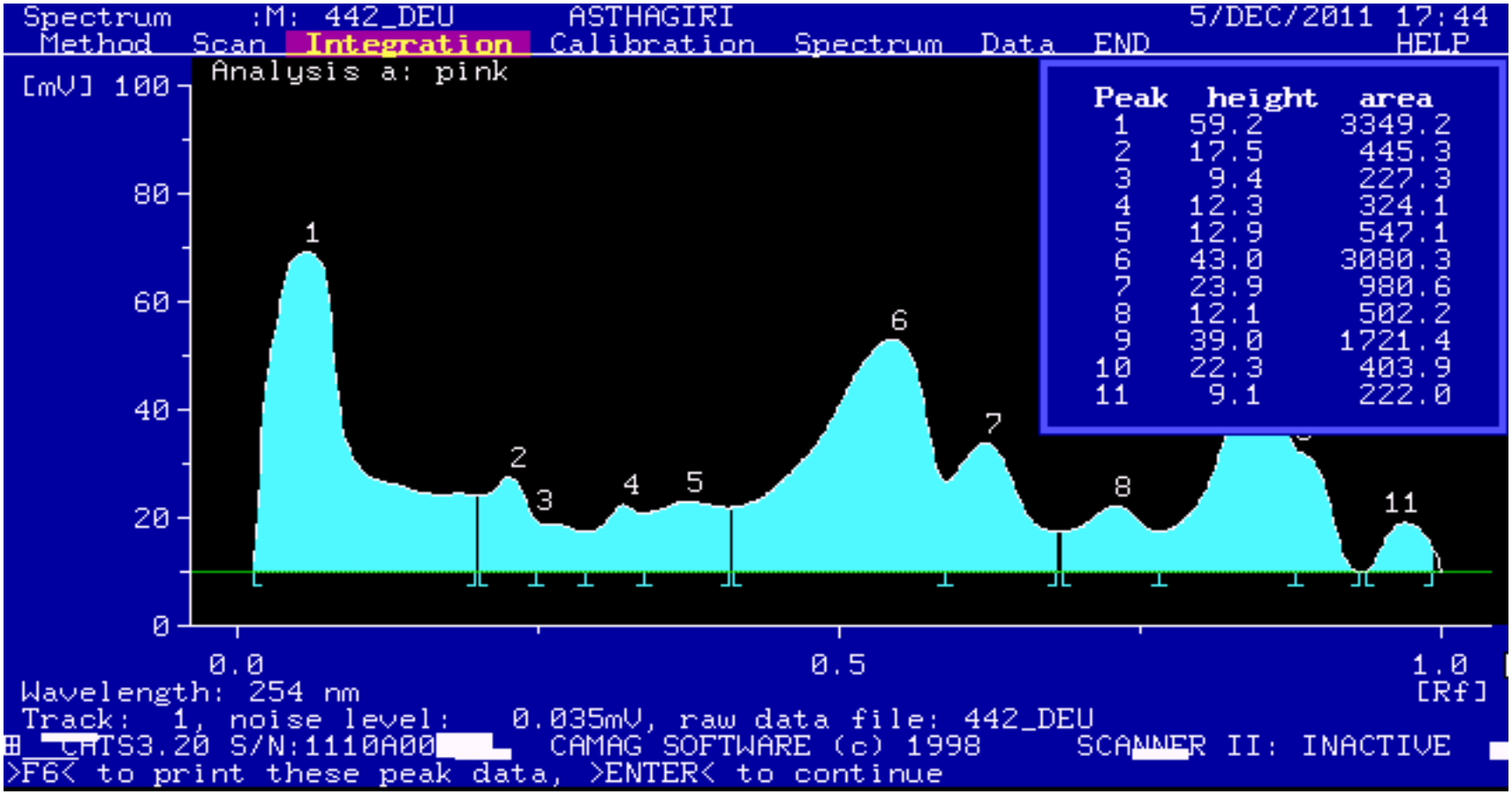


Fig.5.10 HPTLC spectrum in methanolic flower extract of *Hibiscus syriacus*

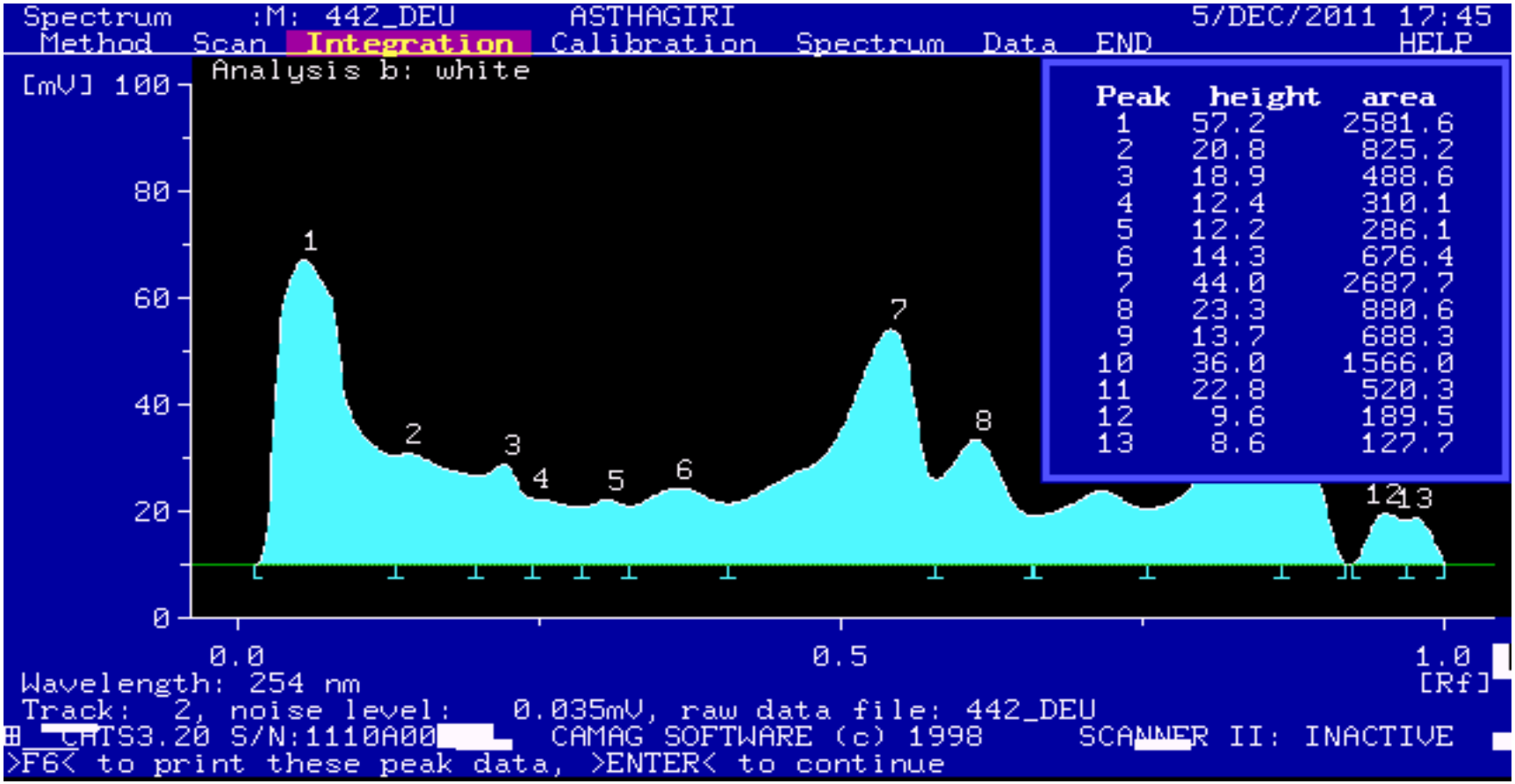


Fig.5.11 HPTLC spectrum in methanolic flower extract of *Hibiscus arnottianus*

5.8 GC-MS analysis in methanolic flower extracts of Hibiscus species:

5.8.1. *Hibiscus rosa sinensis*

GC-MS chromatogram of methanolic flower extract of *Hibiscus rosa sinensis* was shown in Fig.5.15. The total ion chromatogram (TIC) retention time was about 32 min. The most composition of the extract was isolated around the first 27 min of the analysis procedure and condition. Twenty six compounds were identified in flower extract of *Hibiscus rosa sinensis*. The active principles with their retention time (RT), molecular formula, molecular weight (MW) and concentration were presented in Table.5.14 in which 2-amino-9-(3,4-dihydroxy-5-hydroxymethyl-tetrahydro-furan-2-yl)-3,9-dihydro-purin-6-one(29.79%), gamma-sitosterol(13.81%) and 4-cyclopentene-1,3-diol-D_2 trans (11.12%) were present as major compounds and their mass spectra was given in Fig.5.12, 5.13 & 5.14.

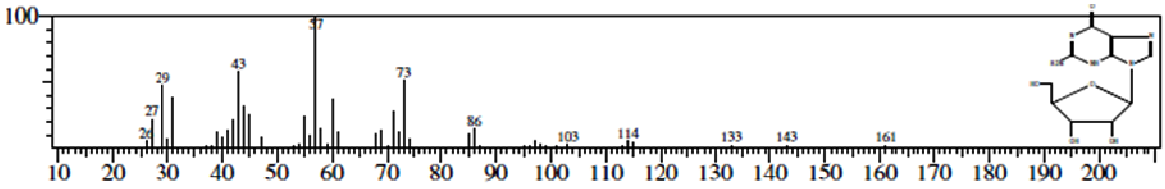


Fig.5.12 Mass spectrum of 2-amino-9-(3, 4-dihydroxy-5-hydroxy methyl-tetrahydro-furan-2-yl)-3, 9-dihydro-purin-6-one

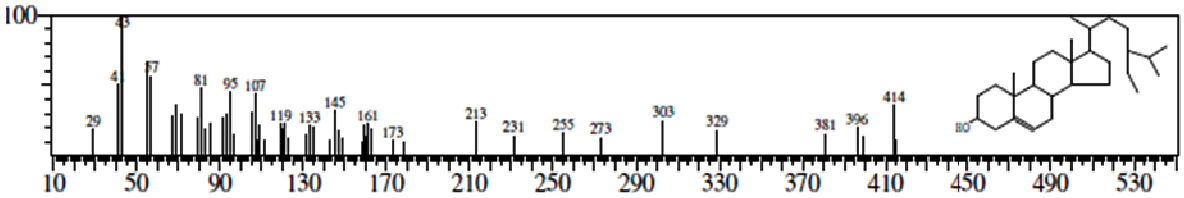


Fig.5.12 Mass spectrum of Gamma-sitosterol

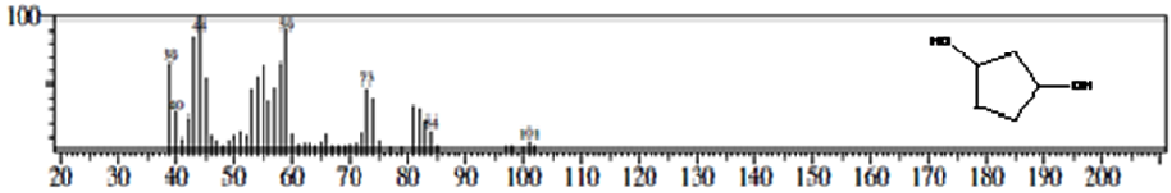


Fig.5.13 Mass spectrum of 4-Cyclopentene-1, 3-diol-D_2, trans

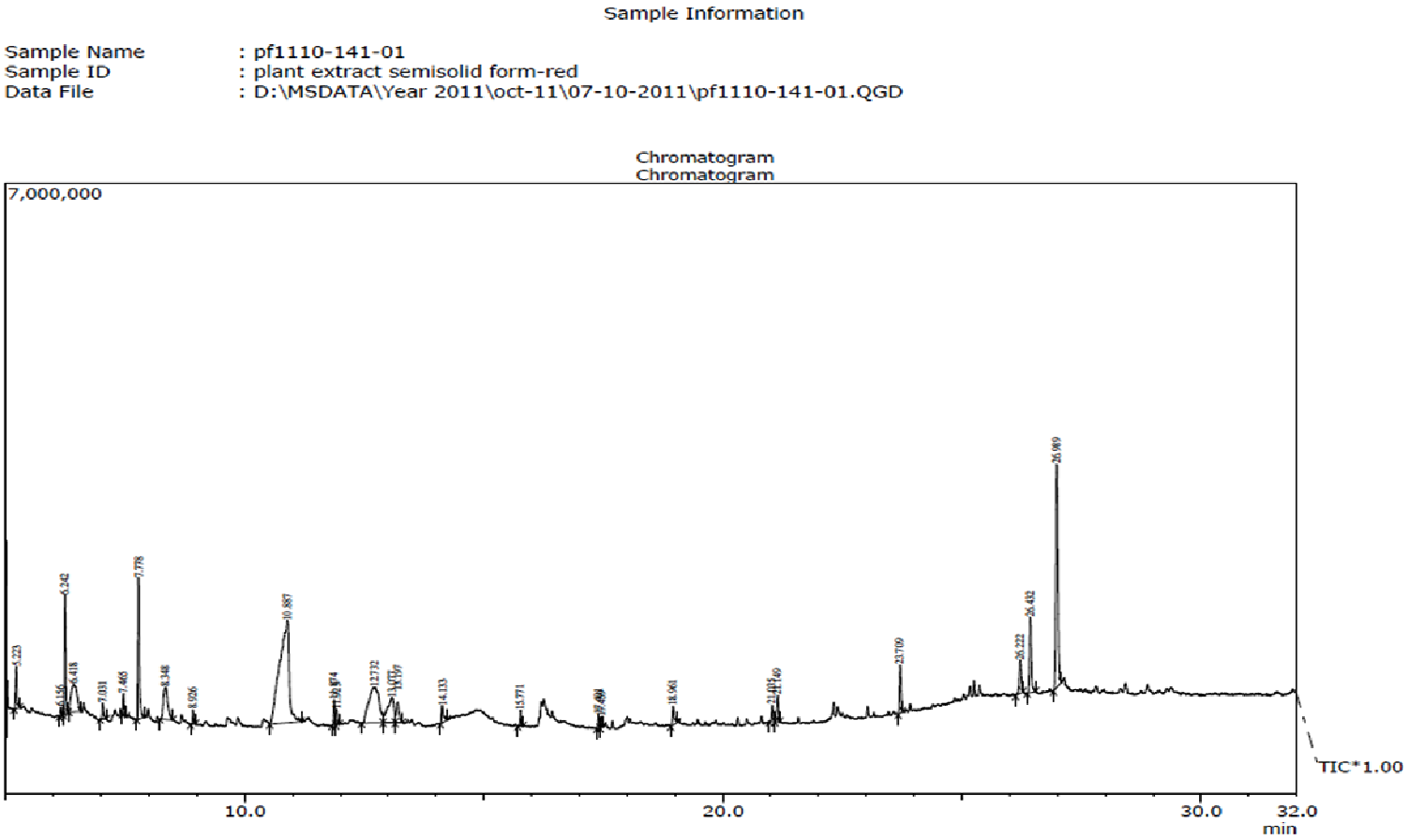


Fig.5.15 GC-MS Spectrum in methanolic flower extract of *Hibiscus rosa sinensis*

Table.5.14. Chemical composition in methanolic flower extract of *Hibiscus rosa sinensis* by GC-MS

S.No	Name of the compound	Formula	MW	RT	Peak area	%Peak area
1.	Maltol	$C_6H_6O_3$	126	5.223	1042721	1.72
2.	2-Acetyl-2-hydroxy-gamma-butyrolactone	$C_6H_8O_4$	144	6.156	193242	0.32
3.	3,5-Dihydroxy-6-methyl-2,3-dihydro-4H-pyran-4-one	$C_6H_8O_4$	144	6.242	2400592	3.95
4.	Glycerin	$C_3H_8O_3$	92	6.418	2946572	4.85
5.	Pyrrolidine	C_4H_9N	71	7.031	430744	0.71
6.	2,3-Dihydro-benzofuran	C_8H_8O	120	7.465	467630	0.77
7.	Acetyl monoglyceride	$C_5H_9O_3$	117	7.778	3616454	5.95
8.	6-Oxoheptanoic acid	$C_7H_{12}O_3$	144	8.348	2509245	4.13
9.	Ethyl-4-chlorobenzoate	$C_9H_9ClO_2$	184	8.926	266261	0.44
10.	2-Amino-9-(3,4-dihydroxy-5-hydroxymethyl-tetrahydro-furan-2-yl)-3,9-dihydro-purin-6-one	$C_{10}H_{13}N_5O_5$	283	10.887	18095546	29.79
11.	Lauric acid	$C_{12}H_{24}O_2$	200	11.874	598091	0.98
12.	4-Methyl-2,5-dimethoxybenzaldehyde	$C_{10}H_{12}O_3$	180	11.923	397810	0.65
13.	4-Cyclopentene-1,3-diol-D_2, trans	$C_5H_6D_2O_2$	102	12.732	6756587	11.12

14.	3-Deoxy-d-mannonic acid	$C_6H_{12}O_6$	180	13.077	2974215	4.90
15.	Mome inositol	$C_{17}H_{14}O_6$	194	13.197	1115572	1.84
16.	n-Capric acid	$C_{10}H_{20}O_2$	170	14.133	618499	1.02
17.	Methyl hexadecanoate	$C_{17}H_{34}O_2$	270	15.771	330868	0.54
18.	Methyl linoleate	$C_{19}H_{34}O_2$	294	17.401	266050	0.44
19.	Methyl petroselate	$C_{19}H_{36}O_2$	296	17.459	193816	0.32
20.	Benzyl beta-d-glucoside	$C_{13}H_{18}O_6$	270	18.961	671949	1.11
21.	2-Monopalmitin	$C_{19}H_{38}0_4$	330	21.035	498436	0.82
22.	Mono(2-ethylhexyl) phthalate	$C_{16}H_{22}O_2$	278	21.149	780488	1.29
23.	1-Eicosanol	$C_{20}H_{42}O$	298	23.709	1335909	2.20
24.	Ergost-5-en-3-ol	$C_{28}H_{48}O$	400	26.222	1219729	2.01
25.	Stigmasterol	$C_{29}H_{48}O$	412	26.432	2619836	4.31
26.	Gamma-sitosterol	$C_{29}H_{50}O$	414	26.989	8390490	13.81

MW-Molecular weight

RT-Retention time

5.8.2. *Hibiscus syriacus*

GC-MS chromatogram of methanolic flower extract of *Hibiscus syriacus* was shown in Fig.5.19. The total ion chromatogram (TIC) running time is about 28 min. The most composition of the extract was isolated around the first 27 minutes of the analysis procedure and condition. Twenty seven compounds were identified in flower extract of *Hibiscus syriacus*. The active principles with their retention time (RT), molecular formula, molecular weight (MW) and concentration were presented in Table.5.15 in which guanosine (29.47%), 1,2,4-cyclopentanetriol (15.38%) and glycerin (10.46%) were present as major constituents and their mass spectra was given in Fig.5.16, 5.17 & 5.18.

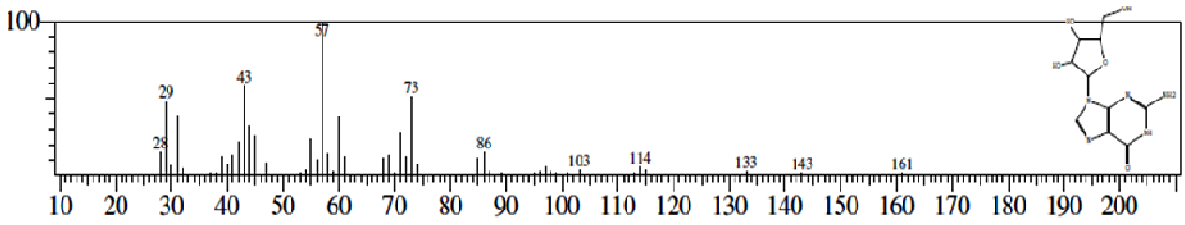


Fig.5.16 Mass spectrum of Guanosine

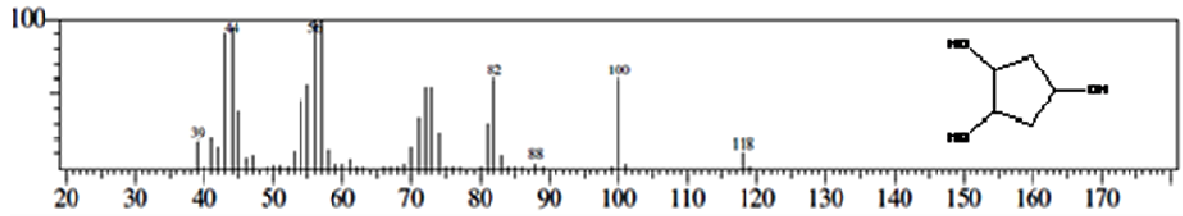


Fig.5.17 Mass spectrum of 1, 2, 4-Cyclopentanetriol

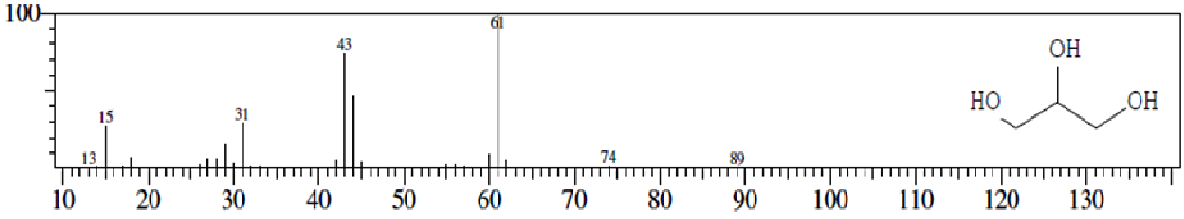


Fig.5.18 Mass spectrum of Glycerin

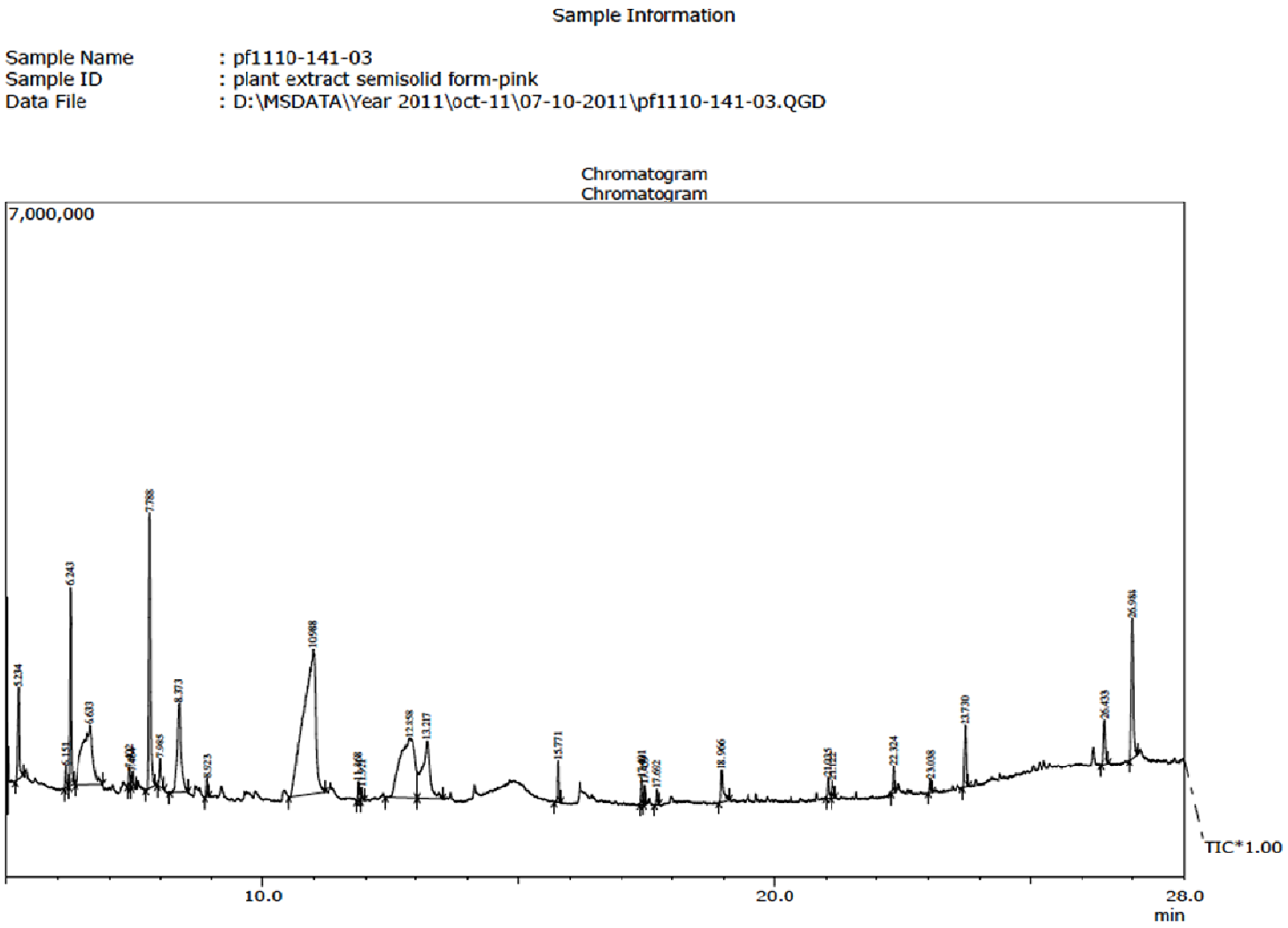


Fig.5.19 GC-MS Spectrum in methanolic flower extract of *Hibiscus syriacus*

Table.5.15. Chemical composition in methanolic flower extract of *Hibiscus syriacus* by GC-MS

S.No	Name of the compound	Formula	MW	RT	Peak area	%peak area
1.	Maltol	$C_6H_6O_3$	126	5.234	2304252	2.51
2.	2-Acetyl-2-hydroxy-gamma-butyrolactone	$C_6H_8O_4$	144	6.151	409189	0.45
3.	3,5-Dihydroxy-6-methyl-2,3-dihydro-4H-pyran-4-one	$C_6H_8O_4$	144	6.243	3719020	4.05
4.	Glycerin	$C_3H_8O_3$	92	6.633	9600881	10.46
5.	Tetrahydrofuran-5-on-2-methanol	$C_{11}H_{16}O_7$	260	7.402	422126	0.46
6.	2,3-Dihydro-benzofuran	C_8H_8O	120	7.464	397621	0.43
7.	Glycerol acetate	$C_5H_{10}O_4$	134	7.788	7172137	7.81
8.	Propyl acetate	$C_5H_{10}O_2$	102	7.985	868156	0.95
9.	6-Oxoheptanoic acid	$C_7H_{12}O_3$	144	8.373	5206684	5.67
10.	Ethyl 4-chlorobenzoate	$C_9H_9ClO_2$	184	8.923	303261	0.33
11.	Guanosine	$C_{10}H_{13}N_5O_5$	283	10.988	27059917	29.47
12.	Lauric acid	$C_{12}H_{24}O_2$	200	11.868	423817	0.46
13.	4-Methyl-2,5-dimethoxybenzaldehyde	$C_{10}H_{12}O_3$	180	11.921	283567	0.31

14.	1,2,4-Cyclopentanetriol	$C_5H_{10}O_3$	118	12.858	14118168	15.38
15.	3-Deoxy-d-mannonic acid	$C_6H_{12}O_6$	180	13.217	6839181	7.45
16.	Methyl hexadecanoate	$C_{17}H_{34}O_2$	270	15.771	763224	0.83
17.	Methyl linoleate	$C_{19}H_{34}O_2$	294	17.401	458689	0.50
18.	Methyl petroselate	$C_{19}H_{36}O_2$	296	17.459	295618	0.32
19.	Methyl stearate	$C_{19}H_{38}O_2$	298	17.692	258287	0.28
20.	Benzylbeta-d-glucoside	$C_{13}H_{18}O_6$	270	18.966	1213172	1.32
21.	2-Monopalmitin	$C_{19}H_{38}H_4$	330	21.035	566798	0.62
22.	Palmitaldehyde	$C_{16}H_{32}O$	204	21.122	470509	0.51
23.	n-Hexatriacontane	$C_{36}H_{74}$	506	22.324	545161	0.59
24.	Docosane	$C_{22}H_{46}$	310	23.038	297370	0.32
25.	Pentatriacontane	$C_{35}H_{72}$	492	23.730	1688595	1.84
26.	Stigmasterol	$C_{29}H_{48}O$	412	26.433	1299718	1.42
27.	Gamma-sitosterol	$C_{29}H_{50}O$	414	26.988	4833753	5.26

MW-Molecular weight

RT-Retention time

5.8.3. *Hibiscus arnottianus*

GC-MS chromatogram of methanolic flower extract of *Hibiscus arnottianus* was shown in Fig.5.23. The total ion chromatogram (TIC) retention time is about 28 minutes. The most composition of the extract isolate around the first 26.98 min of the analysis procedure and condition. Twenty three compounds were identified in flower extract of *Hibiscus arnottianus*. The active principles with their retention time (RT), molecular formula, molecular weight (MW) and concentration were presented in Table.5.16 in which guanosine (31.08%), 1,4-anhydrohexitol (15.44%) and glycerol (8.36%) were present as predominant components and their mass spectra was shown in Fig.5.20, 5.21 &5.22. Major chemical compounds present in flower extracts of Hibiscus species were shown in Fig.5.24.

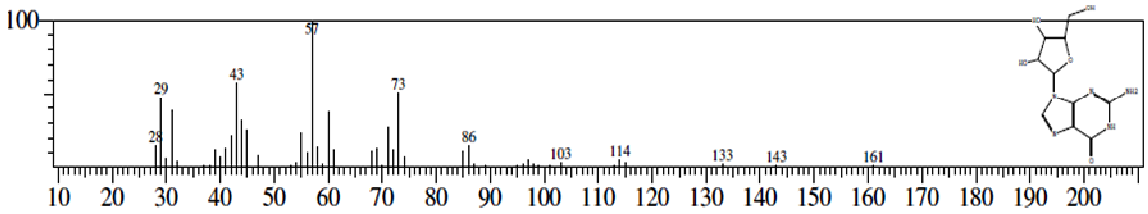


Fig.5.20 Mass spectrum of Guanosine

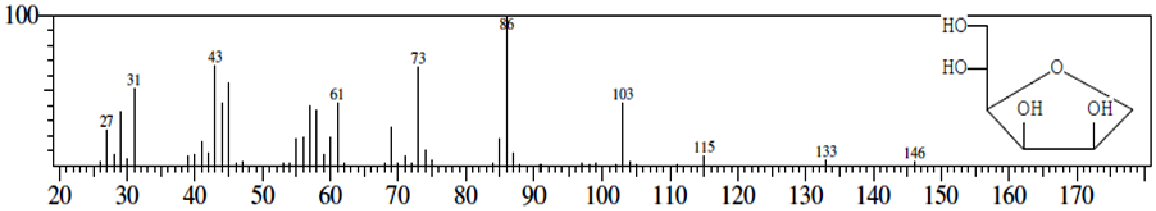


Fig.5.21 Mass spectrum of 1, 4-anhydrohexitol

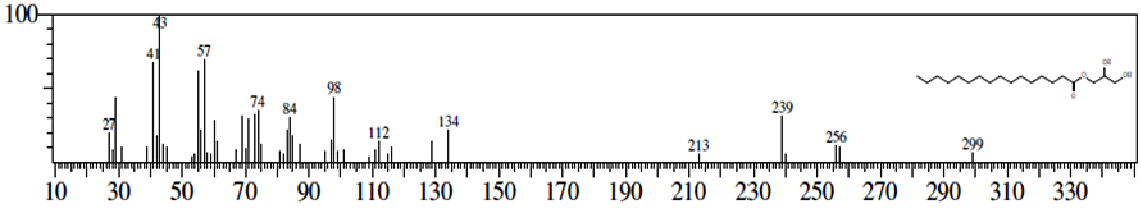


Fig.5.22 Mass spectrum of Glycerol

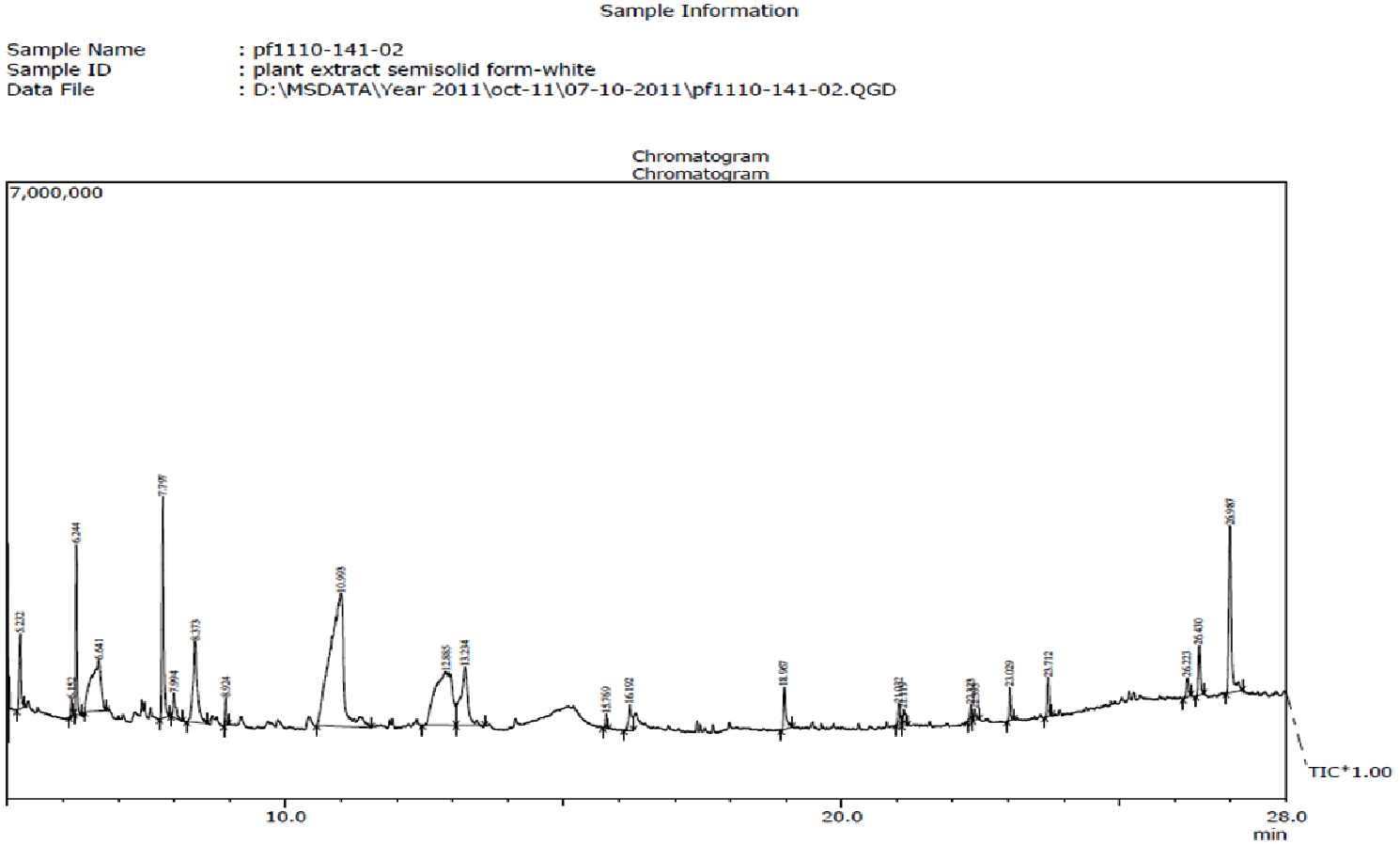


Fig.5.23 GC-MS Spectrum in methanolic flower extract of *Hibiscus arnottianus*

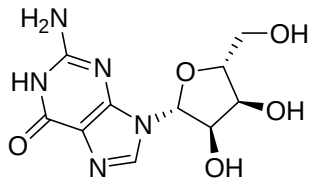
Table.5.16. Chemical composition in methanolic flower extract of *Hibiscus arnottianus* by GC-MS

S.No	Name of the compound	Formula	MW	RT	Peak area	%peak area
1.	Maltol	$C_6H_6O_3$	126	5.232	2041848	2.18
2.	2-Acetyl-2-hydroxy-gamma-butyrolactone	$C_6H_8O_4$	144	6.152	298619	0.32
3.	3,5-Dihydroxy-6-methyl-2,3-dihydro-4H-pyran-4-one	$C_6H_8O_4$	144	6.244	3540202	3.78
4.	Glycerol	$C_3H_8O_3$	92	6.641	7840909	8.36
5.	Acetin	C_9H_{18}	126	7.797	6584009	7.02
6.	Glycol	$C_4H_8O_3$	104	7.994	907462	0.97
7.	6-Oxoheptanoic acid	$C_7H_{12}O_3$	144	8.373	5524701	5.89
8.	Ethyl-2-chlorobenzoate	$C_9H_9ClO_2$	184	8.924	478245	0.51
9.	Guanosine	$C_{10}H_{13}N_5O_5$	283	10.993	29135227	31.08
10.	1,4-Anhydrohexitol	$C_6H_{12}O_5$	164	12.885	14474304	15.44
11.	3-Deoxy-d-mannonic acid	$C_6H_{12}O_6$	180	13.234	6470001	6.90
12.	Methyl palmitate	$C_{17}H_{33}O_2$	269	15.769	297841	0.32

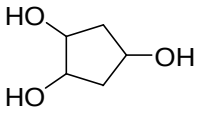
13.	Palmitic acid	$C_{16}H_{32}O_2$	256	16.192	1321374	1.41
14.	Benzylbeta-d-glucoside	$C_{13}H_{18}O_6$	270	18.967	1620577	1.73
15.	2-Hydroxy-1-ethyl palmitate	$C_{19}H_{38}O_4$	330	21.032	680098	0.73
16.	1,2-Epoxynonadecane	$C_{19}H_{38}O$	282	21.119	587421	0.63
17.	Tetracosane	$C_{24}H_{50}$	338	22.323	37702	0.40
18.	Alpha.-glyceryl linoleate	$C_{21}H_{38}O_4$	354	22.395	344925	0.37
19.	Erucylamide	$C_{22}H_{43}NO$	337	23.029	836105	0.89
20.	1-Eicosanol	$C_{20}H_{42}O$	298	23.712	1351662	1.44
21.	Ergost-5-en-3-ol	$C_{28}H_{48}O$	400	26.223	721294	0.77
22.	Stigmasterol	$C_{29}H_{48}O$	412	26.430	1626422	1.73
23.	Gamma.-sitosterol	$C_{29}H_{50}O$	414	26.987	6696078	7.14

MW-Molecular weight

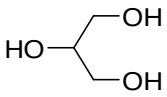
RT-Retention time



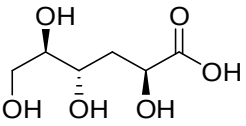
Guanosine



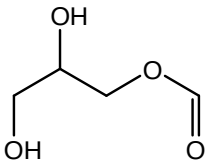
1,2,4-cyclopentanetriol



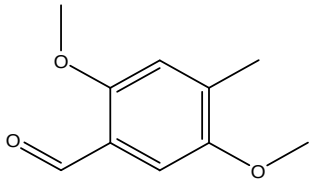
Glycerol



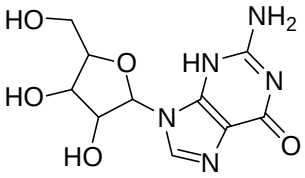
3-deoxy-d-mannonic acid



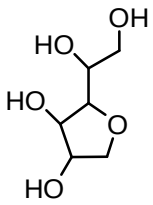
Acetin



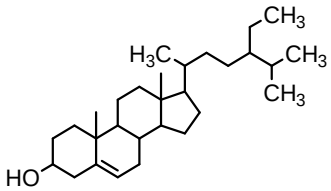
4-methyl-2,5 dimethoxy benzaldehyde



2-amino-9-(3,4-dihydroxy-5-hydroxymethyl-tetrahydro-furan-2-yl)-3,9-dihydro-purin-6-one



1, 4 anhydro hexitol



Gamma-sitosterol

Fig.5.24 Major chemical compounds present in methanolic flower extracts of Hibiscus species

5.7. Evaluation of antimicrobial activity in methanolic flower extracts of Hibiscus species:

The disc diffusion method was used to determine the antimicrobial activity of methanolic flower extracts of three Hibiscus species and shown in Table.5.17. The antibacterial activity of the extracts were tested against bacteria like *S. aureus* & *E. coli* (Fig.5.25 &5.26) and the antifungal activity against *A. niger* (Fig.5.27) in different concentrations and compared with positive controls, ciprofloxacin for antibacterial activity where as ketaconazole for antifungal activity respectively. Flower extracts of all the Hibiscus species exhibited marked antimicrobial activity against the tested microorganisms. The antimicrobial effects of flower extracts of all the tested Hibiscus species against the tested microorganisms were falls in the following order:

S.aureus > *E.coli* > *A.niger*

Among the three Hibiscus species, *Hibiscus syriacus* shows significant activity against gram positive bacteria where as *Hibiscus arnottianus* shows significant activity against Gram negative bacteria and fungi. These effects may be due to presence of flavonoids, tannins, phenolics compounds present in these flower extracts.

Table.5.17. Screening of antimicrobial activity in methanolic flower extracts of Hibiscus species

S.No	Hibiscus Species	Zone of inhibition (mm)								
		S.aureus			***E.coli***			***A.niger***		
		25 µg	100 µg	Std*	25 µg	100 µg	Std*	25µg	100 µg	Std**
1.	***H. rosa sinensis***	18	20.2	30	13	19	26	14	17	25.4
2.	***H. syriacus***	17.3	28.0	30	12	18	26	15	15	25.4
3.	***H.arnottianus***	24	27	30	17	23	26	18.4	22	25.4

Std* - Ciprofloxacin (100 µg /disc)

Std** - Ketaconazole (100 µg /disc)

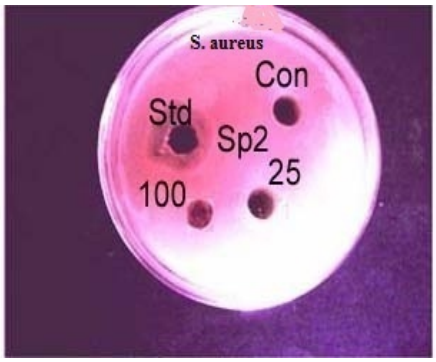


Fig.5.25 Antibacterial activity against *S.aureus*

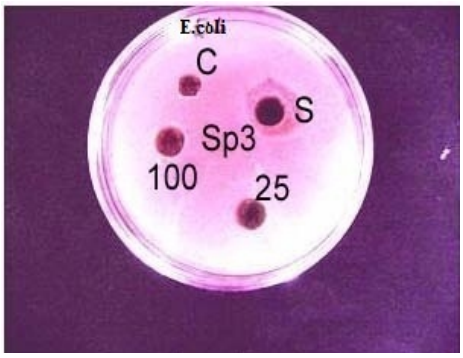


Fig.5.26 Antibacterial activity against *E.coli*

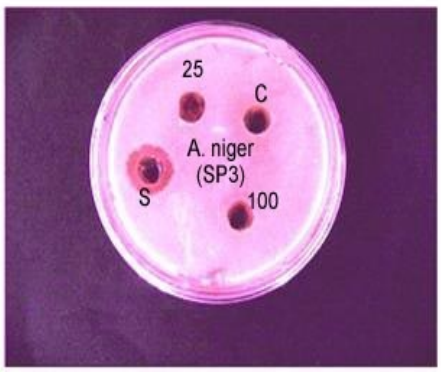


Fig.5.27 Antifungal activity against *A.niger*

SUMMARY

&

CONCLUSION

6. SUMMARY AND CONCLUSION

Medicinal plants containing inherent active ingredients used to cure or prevent the diseases. The use of traditional medicines and medicinal plants in most developing countries as therapeutic agents for the maintenance of good health has been widely observed. So, the identification of bioactive compounds in plants, their isolation, purification and characterization of active ingredients in crude extracts by various analytical methods is important.

The active compounds from the flowers of *Hibiscus rosa sinensis, Hibiscus syriacus* and *Hibiscus arnottianus* were extracted by using petroleum ether, ethyl acetate and methanol and their physical properties were calculated and the extractive value was presented in the following order,

Methanol > Ethyl acetate > Petroleum ether

It was identified that methanol has a stronger extraction capacity which could have produced greater number of polar active constituents. So, in the present study, the further evaluations were made by using methanolic extract of flowers of Hibiscus species.

On qualitative screening of phytochemicals and vitamins in flower of Hibiscus species showed the presence of carbohydrates, protein, glycosides, flavonoids, phenolics, tannins, Steroids, thiamine, niacin, riboflavin and ascorbic acid. The answered phytochemicals and vitamins were subjected for quantitative screening and found that the concentration of flavonoids, total phenolic content, tannins, carbohydrates and protein were present in the following order,

Hibiscus syriacus > *Hibiscus arnottianus* > *Hibiscus rosa sinensis.* In case of vitamins, the concentration of thiamine, niacin, riboflavin& ascorbic acid were more in *Hibiscus rosa sinensis* than *Hibiscus syriacus* and *Hibiscus arnottianus.*

The data obtained from proximate analysis of flower powder of Hibiscus species such as total ash, acid insoluble, water soluble and sulphated ash concluded that the total ash and acid insoluble ash was found to be high in *Hibiscus arnottianus* than *Hibiscus syriacus* and *Hibiscus rosa sinensis* whereas water soluble and sulphated ash were more in *Hibiscus rosa sinensis* than *Hibiscus syriacus* and *Hibiscus arnottianus.*

The inorganic elements were qualitatively analyzed in flower powder of Hibiscus species and found to contain calcium, iron, phosphorus in three species and the other elements such as sodium & potassium were present only in the flower of *Hibiscus syriacus* and *Hibiscus arnottianus*. The data obtained on elemental concentration of the Hibiscus species by atomic absorption spectroscopy were reported. However, the results showed that *Hibiscus arnottianus* had maximum concentration of calcium and phosphorus than *Hibiscus syriacus* and *Hibiscus rosa sinensis* whereas *Hibiscus arnottianus* contains higher content of iron than *Hibiscus rosa sinensis* and *Hibiscus syriacus.*

The detailed analysis for the comparison and standardization of the flowers of Hibiscus species were made by TLC, UV, FT-IR, HPTLC and GC-MS finger printing studies. The results from preliminary phytochemical screening of methanolic flower extract of Hibiscus species were again confirmed by TLC chromatographic profile developed with various mobile phase and visualizing agents.

The UV absorbance peaks of the methanolic flower extract of Hibiscus species were listed in Table.5.10. The methanolic flower extract of *Hibiscus rosa sinensis*

showed one λ_{max} at 272.5 nm where as *Hibiscus syriacus* and the *Hibiscus arnottianus* showed two λ_{max} values at 257 nm & 358.5 nm and 256.5 nm & 351 nm.

The IR spectrum of the methanolic flower extracts of Hibiscus species had the characteristic absorption peak and were reported in Table.5.11, 5.12 and 5.13. The finger print region of the extracts was found to be dissimilar. The IR spectrum of the flower extract of *Hibiscus rosa sinensis* showed about 18 peaks where as the *Hibiscus syriacus* showed 16 peaks and the flower of *Hibiscus arnottianus* showed 15 peaks. The IR spectral data showed the presence of O-H, N-H, C-H, C-N, N-O, S-O and N-N stretchings in all the flower extracts of Hibiscus species. The peaks at 2852, 1107, 890 & 663 were absent in *Hibiscus arnottianus* when compared to *Hibiscus rosa sinensis* and *Hibiscus syriacus.* All the extracts were found to have band between 3400-3200 cm^{-1} showing the presence of O-H group, this confirmed the presence of phenolic compounds which may be responsible for the antimicrobial activity.

HPTLC studies revealed that methanolic flower extract of flower of *Hibiscus rosa sinensis* gives 26 spots on TLC plate whereas *Hibiscus syriacus* and *Hibiscus arnottianus* gives 27 & 23 spots respectively.

The GC-MS analysis represented the presence of 26, 27 & 23 components in the extracts of *Hibiscus rosa sinensis*, *Hibiscus syriacus* and *Hibiscus arnottianus* respectively. The resolved components were tentatively identified by NIST08.LIB, WILEY8.LIB, and FAME.LIB library sources. The chemical composition of the extracts of Hibiscus species showed difference in both quantitative and qualitative aspects.

The *Hibiscus rosa sinensis*, *Hibiscus syriacus* and *Hibiscus arnottianus* consisted mainly of gamma-sitosterol, stigmasterol, and 6-oxoheptanoic acid

respectively as major composition. The constituents such as 3-deoxy-d-mannonic acid, 2, 3-dihydro benzo furan, ethyl 4-chloro benzoate, lauric acid, 4-methyl 2,5dimethoxy benzaldehyde, methyl hexadecanoate, methyl linoleate, 2-monopalmitin and methyl petroselate were found to be common in both *Hibiscus rosa sinensis* and *Hibiscus syriacus.* Two compounds such as ergost-5-enol and 1-eicosanol were common in *Hibiscus rosa sinensis* and *Hibiscus arnottianus*. The only compound called gaunosine was common in *Hibiscus arnottianus* and *Hibiscus syriacus.* The constituents, pyrrolidine, 2-amino-9-(3,4-dihydroxy-5-hydroxymethyl-tetrahydrofuran-2-yl)-3,9-dihydro-purin,4-cyclopentene-1,3diol, mome inositol, n-capric acid and mono(2-ethylhexyl)phthalate were present only in *Hibiscus rosa sinensis* whereas acetin, ethyl 2-chlorobenzoate, 1,4-anhydrohexitol, methyl palmitate, palmitic acid, 2-hydroxy-1-(hydromethyl)ethyl palmitate, 1,2-epoxynonadecane, tetracosane, alpha-glyceryl linoleate, erucylamide and glycerol were present only in *Hibiscus arnottianus.* Tetrahydrofuran-5-on-2-methanol, propyl acetate, 1, 2, 4-cyclopentanetriol, methyl stearate, palmitaldehyde,n-hexatriacontane, docosane and pentatriacontane were present only in *Hibiscus syriacus.*

The data obtained from antimicrobial activity, the methanolic flower extract of *Hibiscus syriacus* and *Hibiscus arnottianus* showed superior antibacterial and antifungal activity respectively due to the presence of the high content of flavonoids and phenolics when compared to *Hibiscus rosa sinensis.*

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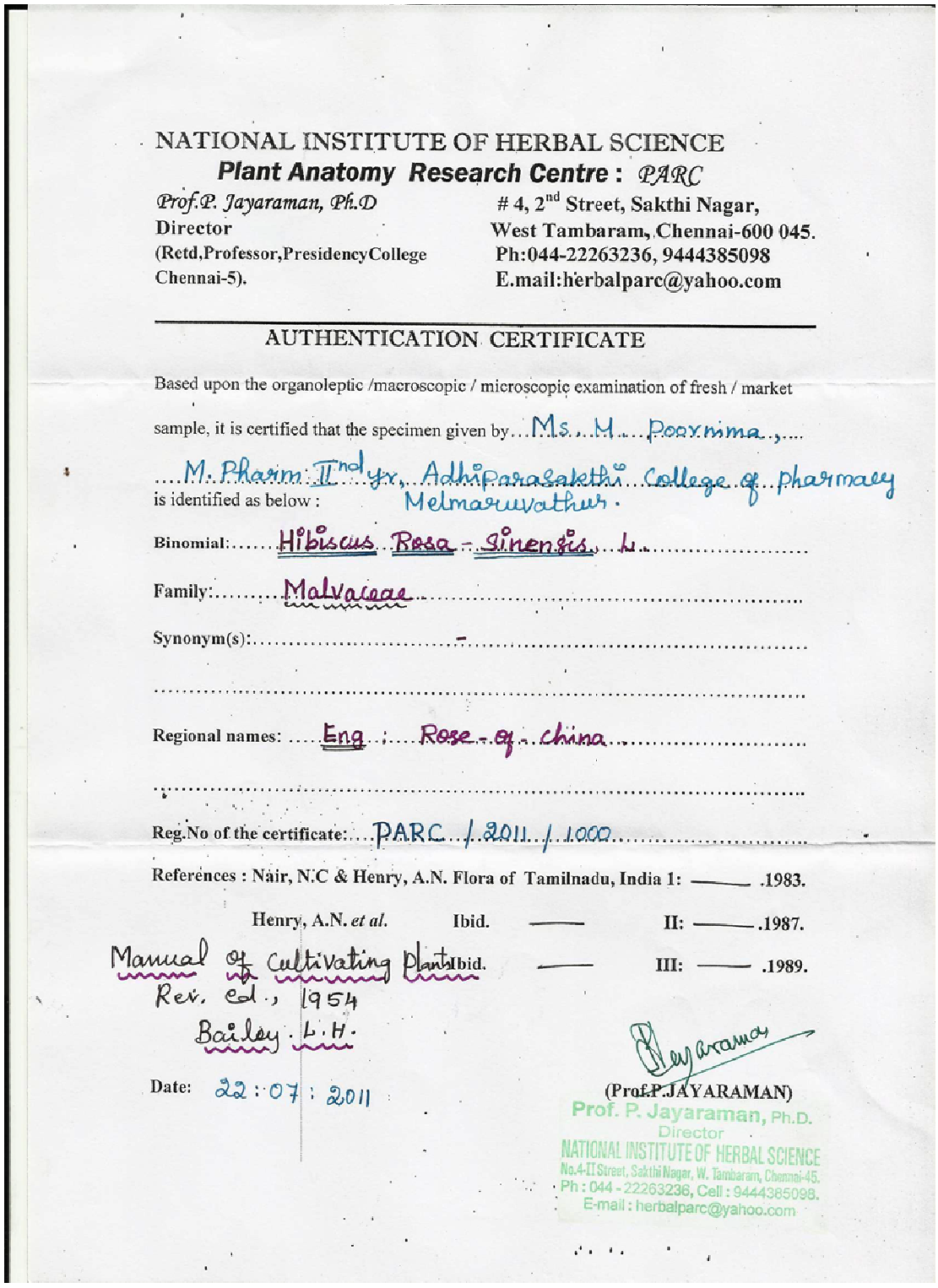
ANNEXURES

8. ANNEXURES



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AUTHENTICATION CERTIFICATE

Based upon the organoleptic /macroscopic / microscopic examination of fresh / market sample, it is certified that the specimen given by Ms. M. Poornima, M. Pharm IInd yr, Adhiparasakthi College of pharmacy Melmaruvathur. is identified as below :

Binomial: Hibiscus Rosa - sinensis L.

Family: Malvaceae

Synonym(s): -

Regional names: Eng : Rose of china

Reg.No of the certificate: PARC / 2011 / 1000

References : Nair, N.C & Henry, A.N. Flora of Tamilnadu, India 1: ——— .1983.

Henry, A.N. *et al.* Ibid. ——— II: ——— .1987.

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Rev. ed., 1954
Bailey. L.H.

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Family:.....Malvaceae.....

Synonym(s):.....–.....

.....

Regional names: .....Eng. : Rose of Sharon.....

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Reg.No of the certificate:.....PARC/2011/1002.....

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AUTHENTICATION CERTIFICATE

Based upon the organoleptic /macroscopic / microscopic examination of fresh / market sample, it is certified that the specimen given by Mr. M. Poornima, M.pharm II yr., Adhiparasakthi College of pharmacy Melmaruvathur. is identified as below :

Binomial: Hibiscus asmottianus, Gray

Family: Malvaceae.

Synonym(s): -

Regional names: -

Reg.No of the certificate: PARC/2011/1001

References : Nair, N.C.& Henry, A.N. Flora of Tamilnadu, India 1: — .1983.

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Rev. ed., 1954
Bailey, L.H.

Date: 22:07:2011.

(Prof.P.JAYARAMAN)

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